Herbicides and their Lethal and Sub-lethal Effects on the Chemical Communication System of *Xenopus laevis*

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Abstract

Amphibian populations are in mass decline on a global scale. Various explanations have been considered, including harmful effects from exposure to toxicants. Using *Xenopus laevis* adults and tadpoles, potential sublethal effects of atrazine, a herbicide, were investigated in this thesis. I also investigated the toxicity of an organic herbicide compared this with the toxicity of a synthetic herbicide, using LC50 values.

Whether *X. laevis* adult frogs could communicate chemically was tested experimentally. The results suggest that adult female *X. laevis* communicate chemically, but there was no evidence that male individuals did so. For testing tadpoles I used a kin-preference assay. An encouraging trend for kin preference was evident, for both an outbred and an inbred line. Tadpoles changed their behaviour after exposure to 10µg/L of atrazine for 24 hr. Kin preferences in the control tests were reversed after exposure. A hypothesis of altruistic kin avoidance was suggested by these results. However, when individuals were isolated and then exposed, these individuals had more pronounced preference for kin compared to controls.

X. laevis tadpoles exposed to Organic Interceptor (organic herbicide) had a LC50 that was more than 7000 times lower than the 20 % recommended dose, whereas Roundup Renews' (a synthetic herbicide) LC50 was around 8 times lower than the 1 % recommended dose.

This research adds to evidence that toxicants have a negative impact on amphibian populations, and suggests that more research needs to be conducted to identify other sublethal effects of toxicants and to clarify the implications these effects might have for the amphibian populations in nature.

Preface

Objectives

My overall objective was to investigate whether sublethal effects of toxicants are harmful to amphibians and to their chemical communication system.

In chapter 1, I go into depth about the causes of amphibian decline, such as diseases, temperature and toxicants. An overview of the mechanism involved with animal communication is discussed, this is then narrowed down to chemical communication, what it is, where and when it is used and the advantages of chemical communication.

Chapter 2 is an experimental study asking the question: do *Xenopus laevis* chemically communicate in both adult and tadpole stages? It seems likely that these animals should chemically communicate due to their habitat, and if so, manipulation of their communication system might allow us to better understand their behaviour and the effect of environmental influences.

Chapter 3 tries to identify the impact of a sublethal dosage of the herbicide atrazine on the chemical communication system of this anuran. This is an important question because it might suggest that disruption of the amphibian communication system might be an unidentified and indirect cause for the decline of amphibians.

Chapter 4 identifies the toxicity of an organic herbicide compared to a synthetic herbicide. This chapter aims to demonstrate how lethal herbicides can be, and also to see whether the toxicity of an organic and a synthetic herbicide differs.

Chapter 5 is a general discussion, tying all of the other chapters together and making conclusions from my findings.

Chapter 1

Chemical Communication

1.1 Amphibian decline

We are witnessing a phenomenon causing global decline in the populations of many amphibian species (Houlahan *et al.*, 2000; Kiesecker *et al.*, 2001). Various reasons have been suggested for this decline, from global warming (Pounds *et al.*, 1999) to parasites and diseases (Daszak *et al.*, 1999), but the emerging consensus is that there may not be any one reason for the decline. We may need a multi-factorial explanation.

Pacific warming may be one of the factors influencing amphibian decline (Kiesecker *et al.*, 2001). Costa Rica has experienced an increase in air temperature (Graham, 1995), accompanied by a decline of amphibians (Pounds & Crump, 1994), suggesting that temperature is negatively affecting the amphibian population. Pounds *et al.* (1999) showed that dry-season mist frequency in Costa Rico, which is associated with the sea temperature, may be negatively impacting on the amphibian populations, and they proposed a dual explanation of decline (the 'climate-linked epidemic hypothesis'). Harlequin frogs (*Atelopus varius)* may gather near a waterfall when their habitat has dried-up and this locality for the frogs may make the frogs more susceptible to attack by parasitic fliers (Pounds *et al.*, 1999). Lips *et al.* (2003) also studied amphibian decline in Costa Rica and agreed with Pounds *et al.*(1999) that the climate appears to be having an adverse indirect effect on the amphibian population. Findings from another study of the decline of amphibians (Davidson et al., 2002), conducted in California, were not as conclusive as those of Pounds *et al* (1999), suggesting that climate changes had only a slight effect on the amphibian population. In California, other

factors, such as agrochemicals, were apparently having a stronger impact (Davidson *et al.*, 2002).

When climate changes, the amount of exposure to ultraviolet-B (UV-B) radiation emitted can be altered (Kiesecker *et al.*, 2001), and several studies have shown an influence of differing amounts of UV-B at different life stages of amphibians (Kiesecker *et al.*, 2001; Davidson *et al.*, 2002; Bridges & Boone, 2003). Effects of exposure to UV-B on amphibians seem to vary depending on size, life stage (Crump *et al.*, 1999) and species (Davidson *et al.*, 2002). UV-B can negatively affect amphibians by causing deformities (Ankey *et al.*, 2000) and by decreasing hatching-success rate (Lizana & Pedraza, 1998). However, Bridges and Boone (2003) showed an increase in the survival of the tadpoles exposed to higher levels of UV-B which was contrary to what Crump *et al* (1999) found. Lastly, UV-B can decrease an amphibian's rate of development and slow its growth (Smith *et al.*, 2000). If the animal develops slowly, it has a higher probability of being attacked by predators and the food resources available to it may become limited due to other animals growing at a normal rate and consuming these resources.

UV-B may break down the immune system of amphibians, although there does not seem to be any direct effects from the UV-B. However secondary influences may accumulate and become lethal. Amphibians may become more vulnerable to pathogens, infections and agrochemicals when these factors are experienced in combination with UV-B radiation (Boone & Bridges, 1999; Kiesecker et al., 2001).

Pathogens appear to be another important cause of the extensive worldwide amphibian decline (Laurance *et al.*, 1996). Drastic numbers of amphibians have died in Australia over

the last 30 years. Laurance *et al* (1996) argued that in Australia the most plausible explanation for extensive decline is an epidemic disease caused by an exotic pathogen. They suggested the pathogen is water-borne and the vectors that are spreading the disease over the country might be birds, fish or aquatic insects, or they might be other frogs and toads. However, Hero and Gillespie (1997) suggested that Laurance *et al.* (1996) were premature in their conclusions, as there has been no identified pathogen and referring to several sick frogs in one area as a widespread epidemic was misleading.

Once its immune system is compromised by stress or some other factor, amphibians tend to be especially prone to other infections and particularly to skin-related infections. The skin of amphibians is semi-permeable, and therefore sensitive to infection or contamination (Stebbins & Cohen, 1995). Three main agents of skin disease have been identified: bacteria, fungi and viruses (Pessier, 2002). Bacterial skin diseases are: cutaneous injuries; 'red leg' syndrome, which is often associated with *Aeromonas hydrophila*; Mycobacteriosis and chlamydophilosis, which are common in African clawed frogs. Some examples of fungal diseases include: saprolegniasis, a term used for infections caused by oomycete water mould, which are opportunistic pathogens; chytridiomycosis, a fungal disease which is thought to be one of the main causes for amphibian decline; and chromomycosis, caused by a fungus found in saprophytes in plant and soil material. Iridovirus infection, a systemic viral skin disease, is mainly found in tadpoles and metamorphosing animals but it can also occur in adults (Pessier, 2002).

Toxicants have been studied and shown to have adverse effects on amphibians and cause their decline. Herbicides, pesticides and insecticides have all been shown to cause deformities, transformation to hermaphrodites (Hayes *et al.*, 2002a; Hayes *et al.*, 2003), death (Smith, 2001), slowed development (Larson *et al.*, 1998), physiological changes (Park & Propper,

2002; Tavera-Mendoza *et al.*, 2002a; Tavera-Mendoza *et al.*, 2002b) and deleterious behavioural changes (Gopal *et al.*, 1981; Park *et al.*, 2001).

Usage of pesticides, herbicides and insecticide is extensive and worldwide. For example, the United States alone, in 1993, used nearly 35,000 tons of atrazine, a herbicide (Sanderson *et al.*, 2001). The worldwide usage of pesticides per annum is nearly 10 billion kg (USA billion) (Aspelin, 1997). Amphibians that are affected by these pesticides are not just those in direct contact with the application of the toxicant, but also amphibians that are downwind of applications, as well as those that reside near the runoff area from a farm (Davidson *et al.*, 2002).

The exposure of amphibians to toxicants alone can have multiple negative effects, and may make the amphibian more vulnerable to stress. The interaction effects of exposure to a pesticide and an increase in stress (e.g., predator-induced stress) may increase the probability of mortality by a factor of two to three (Relyea & Mills, 2001). A toxicant and an increase in temperature or UV-B may also increase mortality (Boone & Bridges, 1999), although some caution is appropriate here because Bridges and Boone (2003) failed to replicate this finding. However, the potential for the interaction of multiple toxicants to increase the impact on mortality and potential development of amphibians is of serious concern (Boone & James, 2003).

Sublethal concentrations of toxicants and the impact of these on amphibians have been widely studied. Ecologically relevant levels of toxicants are generally at a sublethal level, but dilution from rain or through runoffs means that the sublethal effect might, in the long run, be just as harmful to a population or community as a lethal dosage. Sublethal dosages can affect fertility

(Tavera-Mendoza *et al.*, 2002a; Tavera-Mendoza *et al.*, 2002b; Hayes *et al.*, 2002a), development (Larson *et al.*, 1998; Howe *et al.*, 2004) and endocrine function (Park *et al.*, 2001; Hayes *et al.*, 2003).

Hayes *et al.* (2002a, 2002b, 2003) studied the sublethal effect of atrazine (herbicide) on amphibians and found hermaphroditism, a decrease in the laryngeal size in males, retarded gonadal development, including males growing oocytes, and a ten-fold decrease in testosterone levels.

Park *et al.* (2001) has also studied the sublethal effects of a toxicant, endosulfan, an insecticide, on amphibians. They discovered the amphibian endocrine system was disrupted, mate choice was impaired and the use of the olfactory system was disrupted (c.f Park & Propper, 2002).

All of the sublethal effects these toxicants have on the amphibians are not necessarily directly killing them. However, in the long term, they appear to be affecting the whole community, including other trophic levels (Hildebrand *et al.*, 1980). Some important questions remain. Does decrease in fertility or disruption of the gonads causes significant reduction in reproductive success? Does endocrine disruption and impairment of the olfactory system lead to miscommunication internally and externally, leading to a population decline? Can amphibians that have been exposed to toxicants perceive conspecific cues correctly, or do they misinterpret the communication?

1.2 Overview of Animal Communication

Anurans are known to communicate in four different ways: visually (Hodl & Amezquita, 2001), seismically (Narins, 1990; Narins, 1995), acoustically (Doherty, 1994; Phelps & Ryan, 2000; Hodl & Amezquita, 2001; Waldman, 2001) and chemically (Lee & Waldman, 2002; Waldman & Bishop, 2003). Some anurans use all of these modalities, whilst others appear to be restricted to one or two of these modes.

Depending on the anuran species visual signalling may be used to warn off predators, for signalling the presence of potential prey and as part of the courtship behaviour (Hodl & Amezquita, 2001; Rosenthal *et al.*, 2004). Visual displays may be based on the variations of colours of the skin, and also on movement of the body and limbs (Hodl & Amezquita, 2001).

Vocalization is an especially common form of communication in anuran and it is generally sexually dimorphic (Wilczynski & Chu, 2001). Vocalization is often preliminary to reproduction and for the male is the key to obtaining a mate (Tobias *et al.*, 2004). The male makes calls that inform the female where he is and also to assert his position amongst the other male competitors (Tobias *et al.*, 2004). In some frogs, the female replies, indicating she is either receptive or not. *Xenopus laevis* females are an example. The female of this species indicate that she is receptive by using a special call - 'rapping'. The male respond by continuing to call and by approaching her. If the female of *X. laevis* is unreceptive, she may make another call ('ticking') that informs the male of her mating status (Tobias *et al.*, 1998). The *X. laevis* male's vocalization not only communicate the male's sexual receptivity but also his level of fitness (Emerson, 2001).

Seismic communication is an extension of vocalization (Narins, 1990; Narins, 1995): the vocal sac is filled with air and placed on the ground. The signals made in this way are not only felt but also heard (Narins, 1990).

1.3 Chemical communication

Chemical communication, based on pheromones, is used by some amphibians to identify a home range or territory of an individual and to convey the signaller's sex and size (Madison, 1975; Dawley, 1984; Horne & Jaegar, 1988; Mathis, 1990). Scent marking pheromones are not used to literally repel the intruder from another animal's home area, but rather to identify boundaries and convey information about the resident (Simons *et al.*, 1997; Gautier & Miaud, 2003). The information conveyed in the markings can be used for mate choice.

Plethodon cinereus females may use the markings to learn the size of the male occupying the territory and to learn if he is comparatively large. The female responds by staying in close proximity to the large male and moving away from small males (Mathis, 1991). Home range size can vary and is not necessarily related to the size of an individual, nor is it generally gender based (Kleeberger & Werner, 1982).

Fisher (1954) coined the term 'dear enemy', which translates into animals becoming more aggressive with communication from a stranger than by a territorial neighbour. Once territories have been established, those neighbours pose little threat, and only a defensive motion is required, for example a display. The red-backed salamander (*Plethodon cinereus*) uses pheromonal marking and a number of physical displays to warn off neighbours without the cost of fighting and potential injury (Jaegar, 1981; Jaegar, 1984). Both the male and female of the red-backed salamander actively defend a territory. The animal that initiates the

contest is not always the larger animal, due to fighting being more dependent on speed, instead of strength (Jaegar, 1981).

Several species use faecal pellets as territorial markers, *Plethodon vehiculum* being a well studied example (Ovaska & Davis, 1992). Females of the red-backed salamander, *P. cinereus,* seem to be more interested in faecal pellets than males are, possibly because the territory is especially important for the female's survival and reproductive success (Horne & Jaegar, 1988). For example, females may spend more time nose-tapping the pellets and even squashing them. Horne and Jaeger (1988) found that the female's interest is shown by the amount of time she spends investigating faecal pellets and by her behavioural response, such as submissive or aggressive postures. *P. cinereus* may use chemical communication to outline the boundaries of the home ranges in which they prefer to stay except during the breeding season (Tristram, 1977). These male salamanders also avoid unfamiliar conspecific territories and they identify their own territory from unfamiliar conspecifics using pheromonal signals (Tristram, 1977). Preference for own territory was demonstrated by the males spending more time in burrows self marked by their own faecal pellets than in burrows pellet marked by conspecifics (Jaegar *et al.*, 1986). These territorial pheromones appear to be produced by glands near or in the cloaca (Simon & Madison, 1984). Jaeger and Gabor (1993) found that in *P. cinereus* the intruder and the territorial male resident use the postcloacal gland of the other salamander to distinguish friend from foe.

The native New Zealand frog *Leiopelma hamiltoni* distinguishes between another conspecific's substrate and its own substrate (Waldman & Bishop, 2003). *L. hamiltoni* also responds to the faecal matter of a conspecific as a signal, and is deterred by the faeces if the occupier is larger than the intruder (this is relayed in the faeces) (Lee & Waldman, 2002). It

may be advantageous for amphibians to recognize different conspecifics and then reduce time spent in aggression towards other territorial amphibians in their region (McGavin, 1978).

During the non-breeding season, some amphibians migrate away from their breeding pond. When the breeding season arrives again, they find their way back. Chemical cues have been shown to help guide frogs (Madison & Shoop, 1970) back to their breeding ponds (Grubb, 1973). Reliance on visual cues (Madison, 1969) and use of a celestial compass (Landreth & Ferguson, 1967) are two mechanisms that may aid homing, but reliance on olfactory cues is probably more important in mist species.

Tracy and Dole (1969) induced anosmia in *Bufo boreas* and *Rana clamitans* and the anosmic individuals experienced difficulty orienting back to their breeding sites (Oldham, 1967). Young frogs (*Rana lessonae*) relay on a mix of different odourants to discriminate between strange pond water and their native pond water (Ogurtsov & Bastakov, 2001). Ogurtsov and Baskakov (2001) investigates the sensitivity of the olfactory system in *Rana lessonae,* and showed that laboratory reared frogs during larval development could remember different stimuli dissolved in water, natural and artificial.

Homing by amphibians not only involves the use of pheromones but also the ability to learn. The amphibian has to remember particular odours from specific places, whether it be something the animal deposited itself or something unique to the area (Madison, 1969)

The vomeronasal organ is one of several divisions in the tetrapods' nasal cavity. It is additional to the main olfactory system. In anurans it is situated in the medial position of the oral cavity (Bertmar, 1981). *Plethodon cinereus,* a plethodontid salamander, has nasolabial

grooves and it has been suggested that these structures deliver chemicals to the vomeronasal organ and not to the olfactory organ. This specialized mechanism is not found in other salamanders (Dawley & Bass. 1988). Caecilians also have mechanisms that are based on their tentacle which aids the vomeronasal chemoreception. A tentacular duct carries odourants, running straight to the vomeronasal organ (Schmidt & Wake, 1990). In fish, the olfactory tract is possibly the less dominant receptor for pheromone-mediated signalling and response. Demiski and Northcutt (1983) discovered the terminal nerve (TN), as opposed to the olfactory tract, was used for the response to sex pheromones. The TN is associated with the vomeronasal system and the olfactory system throughout development and is also associated physiologically (Dawley, 1998). Further studies on the TN suggested in vertebrates it could be linked to reproductive behaviour. This is due to gonadotropin-releasing hormone (GnRH) being related to the vertebrate reproductive cycles, and also to the TN. Propper and Moore (1991) found that in females of *Taricha grandulosa* exposed to the mating behaviour of males had an increased amount of GnRH-immunoreactive present in the TN area.

Any odourant that intermingles with the vomeronasal receptors, or the main olfactory receptors, must diffuse through a mucus layer. This mucus layer covers the receptor mucosa and then it has to be absorbed (Dawley, 1998).

An example of an odourant is male courtship pheromone. These are very common in salamanders. Male courtship pheromone actually improves the female's receptivity to mating and its action is mediated through the vomeronasal system (Houck *et al.*, 1998). Work on mammals may be relevant, in female meadow voles, the male odour is sensed through the vomeronasal system and induces estrous synchrony, activates ovarian and uterine growth in reflex ovulating species, accelerates puberty and blocks pregnancy in freshly inseminated females (Lepri & Wysocki, 1987). The different behavioural responses elicited by the

vomeronasal system due to the gendered pheromone could be the result of sexual dimorphism (Dawley, 1992b). In similar sized male and female plethodontid salamanders, the male's olfactory and vomeronasal organs are larger than the female's. It is always the male that performs nose-tapping during courtship (Dawley, 1992a).

The chemical properties of these pheromones have been studied. The primary pheromone isolated in the salamander *P. jordani* is a protein ~22kD (Rollmann *et al.*, 1999). Males of *Cynops pyrrhagaster,* a newt, produce a decapeptide (SIPSKDALLK-OH, called sodefrin). This is a water-soluble peptide, and is secreted by or through the abdominal gland of the cloaca (Kikuyama *et al.*, 1995a; Kikuyama *et al.*, 1995b). The magnificent tree frog, *Litoria splendida*, releases a pheromone, named splendipherin, which is also a peptide (Wabnitez *et al.*, 1999). These studies provide evidence that amphibians do produce mating pheromones.

Chemical communication is important in courtship and mating in amphibians. Females that are sexually receptive and ready to mate broadcast to potential males. The signals of receptivity can be a vocalization or in some cases a pheromonal secretion or sex attractant. The amphibian females may secrete the sex attractant and then wait for the males' response. A male's behavioural response to the sex attractant include agitation, calling and attempts to clasp the female (Rabb & Rabb, 1963). Once the female approves of the males' attribute, the mating process begins.

Amphibian males (especially in salamanders) send out a courtship pheromone once the initial courtship interaction is in progress. This pheromone influences the receptiveness of the female and is very common is salamanders. These courtship pheromones are produced in sexually mature males at the onset of the breeding season, with the glands being only

developed during this period (Houck *et al.*, 1998). The time spent performing courtship rituals is significantly reduced when the females are exposed to this pheromone. Also the female is less likely to leave the courting male (Houck *et al.*, 1998; Rollmann *et al.*, 1999). This is a clear example of sensory reception by the vomeronasal organ.

An advantage of using pheromones is that the signal can be sent out immediately and the behavioural response is rapid. There are different ways the pheromone can be dispersed. Secretion from the cloaca and from skin glands is especially common (Simon & Madison, 1984; Houck et al., 1998; Wabnitez et al., 1999; Rollmann et al., 1999; Pearl et al., 2000). Another mode is by the use of special teeth to inject the pheromone into the female's circulatory system. For example, in the mountain dusky salamander (*Desmognathus ochrophaeus*), the male bites the female first and then rubs his chin gland into the wound to send the courtship pheromone into the circulation system of the female. Injection can make the response immediate, stimulating the female to become fully receptive and to assume the next position of the mating ritual (Verrell, 1988; Houck & Reagan, 1990;). Pheromones may also be circulated in water by the amphibian moving its tail, creating a current. This method is used by the male to project the pheromone to the female and stimulate mating (Halliday, 1975). Another mechanism of pheromonal circulation is depositing pheromones in the faecal pellets (Jaegar et al., 1986;Walls et al., 1989;).

Different mechanisms are used for recognition: familiarity, allele matching, spatial and phenotype matching. Recognition by familiarity entails using prior knowledge of a group of animals, this method being the major mechanism used for individual recognition. There has to be a 'common badge' (e.g., the same food source produces the same odour). Modes of familiarities include the cues emitted, colour patterns or calls and these can either be

specialized or unique. Allele matching is a mechanism used to identify close kin. There is no template learnt, but rather there is linking to the phenotypic cues. At the other extreme, spatial location is the most straightforward mechanism used for recognition. For this, only minimal spatial memory and learning are required, but this mechanism is subject to deceit by others. Yet another mechanism for recognition is phenotype matching, this being used to identify genetic similarity. Given that phenotype and genetic similarity are linked, the subject can recognize kin from nonkin without prior experience. The subject must compare either its own template or that of a relative or a familiar individual with that of the animal in question (Waldman, 1981; Bradbury & Vehrencamp, 1998).

Chemical communication is used by amphibians to distinguish conspecifics from heterospecifics. Most salamanders discriminate between their own odour and a conspecific's, between conspecific's and heterospecific's odours, and between male and female odours (Madison, 1975; Simon & Madison, 1984; Uzendoski & Verrell, 1993). With the onset of the breeding season, odour preference changes, possibly due to the odour chemically changing or because the salamander becomes more aware of the odour (Verrell, 1989). Verrell (1989) hypothesized that males need to be exposed to suitable chemical cues to initiate courtship and found these were only present in intraspecific pairs (Verrell, 1989).

It is very important for males to be able to distinguish conspecifics from heterospecifics, especially when seeking a mate. Pheromonal recognition enables the male to breed with a 'non relative' female and produce outbred offspring (Blaustein & Waldman, 1992). A chemical communication system that aids with recognition could function as a reproductive isolating mechanism (Simon & Madison, 1984; Dawley, 1987). The production of outbred offspring is important because this enables the individual to have offspring with a better

probability of being healthy, being able to fight disease and infection, and to find a mate (Reed et al., 2003).

Tadpoles of some anurans aggregate in kin groups, and there have been many studies of kin recognition and preference by tadpoles. Ability to recognise kin can increase the inclusive fitness of an individual. Recognising kin and schooling can increase the amount of food available, due to a large number of tadpoles swimming around stirring up the substrate and thereby making the food more easily accessible. Non-kin tend to be competitors for food or space and as a group of related tadpoles can deter these intruders from staying. Kin recognition is also related to predator avoidance. When a kin member is injured by a predator, it may release a chemical that warns the rest of the brood (Hews & Blaustein, 1985; Hews, 1988). This alarm response enables tadpoles to avoid the area and thereby avoid the predator. Another benefit of aggregation and kin recognition is potentially aposematic colouration defence (Waldman & Adler, 1979). A group of animals with the same aposematic colouration can be seen more easily by a predator than a single individual.

Kin recognition occurs after metamorphosis in some amphibian species. Blaustein *et al* (1984) tested post-metamorphosed frogs for kin preference and found a strong preference for close association with kin, but Waldman (1986) could not replicate this finding. For frogs, recognising kin aids with breeding, whether it be inbreeding or outbreeding. Some salamanders recognise kin and some even recognise gender, and these abilities help them communicate with conspecifics and heterospecifics (Madison, 1975; Jaegar & Gergits, 1979; Dawley, 1984; Anthony, 1993). When larvae of the American toad *Bufo americanus* were given the choice, using a Y-maze tank, between flowing water from kin and nonkin the subject favoured the kin. When given the choice between blank flowing water and nonkin

flowing water, the subject favoured the blank water source (Waldman., 1985). These experiments illustrate how the emission of a chemical cue enables these larvae to make the distinction between kin and non-kin. In *Rana cascadae* the tadpole's kin recognition ability is even more precise. These tadpoles discriminate between and prefer to associate with full siblings and half siblings, and prefer associating with half siblings instead of non siblings (Blaustein & O'Hara, 1982). Waldman (1981) showed this previously in *Bufo americanus*. These finding imply that these tadpoles use phenotypic matching because the tadpole can use either paternal or maternal origin cues to discriminate between siblings and non siblings (Blaustein & O'Hara, 1982).

1.4 Study animal

My study animals were South African clawed frogs, *Xenopus laevis*. Although native to Africa, these are now also found in areas of the United Kingdom, Mainland Europe, and North America (Tinsley & McCoid, 1996; Tinsley et al., 1996). The body length of an adult female can exceed 10 cm and the adult male generally ranges between 10-30% smaller (Tinsley *et al.*,1996). The tympanic disc is also sexually dimorphic, the male's tympanic being larger (relative to skull size) (Elepfandt, 1996). The tadpoles are efficient filter feeders while the adults will eat anything from tadpoles to small vertebrates (Tinsley *et al.*, 1996). Adults, as well as the tadpoles, are fully aquatic. *Xenopus* sometimes migrate long distances over land, but how often they migration is unclear. However, nocturnal excursions onto land are regular occurrences. The ability of *Xenopus* to aestivate for many months at a time without water in the wild (Tinsley *et al.*, 1996) and move to waterways that do not have water connections elsewhere potentially threatens other frog species, including the New Zealand frog fauna.

Xenopus's predators have not been well documented, but they are probably mainly the same predators that prey on fish. *Xenopus* has a specialised potential defence against predators, toxicants present in skin secretions (Tinsley et al., 1996).

Xenopus's sensory system appears to be adapted for turbid, dark conditions. Their vision is poor, and vision seems to be of limited importance for these frogs that live in turbid water and move mostly during the dark hours (Elepfandt, 1996). The eyes of *Xenopus* are situated on top of the head and have evolved for use on top of or out of the water (Elepfandt, 1996). *Xenopus*'s eyes function well for detection of aerial predators, as has been shown it laboratory conditions with an object being passed rapidly over a tank of *Xenopus*. Their behaviour was to hide for cover, whereas when the object was moved by the side an escape reaction this was rarely seen (Elepfandt, 1996).

Xenopus has another sensory perception system, the lateral line system. The lateral line is a superficial organ that stands free on the skin, is present in tadpoles and in adults. Its function is to detect and analyse water movement and orientation. There are around 180 lateral line organs, situated on and behind the head region and on the trunk (Elepfandt, 1996).

Chemoreception is the last primary sensory system. It is used in mating (Rabb & Rabb, 1963) and prey detection. Rabb and Rabb (1963) demonstrated that the odour of a mating pair of Surinam toads excites unmated males when placed in the mated pair's water. They suggested that an odour excreted by the female elicits this response. *Xenopus* may be able to detect water quality using their chemoreception (Elepfandt, 1996).

Both the male and the female *Xenopus* vocalise (Tobias et al., 1998; Tobias et al., 2004). The male advertisement call is the most common. This call signals his sexual state. Inter- and intraspecific competition calls have also been documented (Tobias et al., 2004). The female has only two calls and these are both related to mating. When the female is receptive to mating, a rapping call is made, stimulating the male. If the female is unreceptive, a ticking call is made after which the males generally do not attempt to amplex (Tobias et al., 1998).

Xenopus laevis has been studied intensely for physiological, immunological and cellular reasons. Also toxicological studies have been conducted on them, including studies by Hayes *et al.* (2002a; 2002b; 2003) and Tavera-Mendoza *et al.* (2002a; 2002b). These are an easy animal to work with and are easily bred. In one breeding, a pair can produce over a thousand larvae in ideal laboratory conditions (personal observation). They become sexually mature after only eight months post egg stage (Tinsley et al., 1996). They are extremely tolerant. For example they can live in almost any kind of water. They survive well with or without vegetation, under low pH, or under high pH (as high as 9) and in the presence of high amounts of lime (Nieuwkoop & Faber, 1994).

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Chapter 2

Chemical Communication

2.1 Introduction

There are four different modalities of communication in anurans: visual (Hodl & Amezquita, 2001), seismic (Narins, 1990; Narins, 1995), acoustic (Hodl & Amezquita, 2001; Doherty, 1994; Waldman, 2001; Phelps & Ryan, 2000) and chemical (Lee & Waldman, 2002; Waldman & Bishop, 2003). In any one instance, the modality of communication may be several of these mechanisms. These different modalities of communication are used in courtship rituals (Tobias et al., 2004), to signal potential prey (Hodl & Amezquita, 2001), to warn off predators (Hodl & Amezquita, 2001), to mark territories, to identify individual fitness and to identify gender (Horne & Jaegar, 1988; Madison, 1975; Mathis, 1990; Dawley, 1984).

Pheromones are the signals used in chemical communication which is especially important in marking home range or territories (Horne & Jaegar, 1988), in mediating mate choice (Horne & Jaegar, 1988), in communicating the size of an individual (Mathis, 1991), in advertising the presence of predators (Tristram, 1977) and in alarms signals (Hews, 1988; Hews & Blaustein, 1985). Amphibian pheromones are excreted as components of faecal pellets (Ovaska & Davis, 1992) (Lee & Waldman, 2002) and urine (Lee & Waldman, 2002), and or liquid secretions from glands (Simon & Madison, 1984).

Chemical communication is especially effective for homing (Tracy & Dole, 1969) and chemical cues sometimes aid amphibians in finding their way back to their breeding ponds after migration (Grubb, 1973; Madison, 1969). Pheromones are also important in ensuring mating success in amphibians. Once a courtship interaction is in progress, courtship

pheromones are often released by the male. The female's receptiveness is affected by the male's pheromones and the male's pheromones encourage the female to stay with the male and not to re-mate. The time spent in a courtship ritual may be dramatically reduced when there is exposure to a pheromone (Houck et al., 1998; Rollmann et al., 1999).

Discriminating conspecifics from heterospecifics decreases the chance of producing inbred offspring, which in turn increases the probability of the offspring being healthy and strong (Dawley, 1987; Simon & Madison, 1984). Pheromonal recognition facilitates outbreeding (Blaustein & Waldman, 1992).

Chemical communication underlies for kin recognition and kin preference, and kin recognition can increase the inclusive fitness of an individual. Kin recognition occurs in tadpoles (Waldman, 1981) and many advantages for tadpoles have been identified. Recognising kin and schooling can increase the amount of food available to the individual tadpole. This is because a large number of tadpoles swimming around may stir up the substrate, making food more accessible. A nonkin tadpole can be recognised as a competitor for food and space and, a group of kin, can deter the intruder from joining the area.

Predator avoidance is an important survival trait. When a kin member is injured by a predator, it may release a chemical that alerts the rest of the brood to danger (Hews, 1988; Hews & Blaustein, 1985). This alarm response means that tadpoles can avoid those areas in which the predator is likely to be encountered. Aposematic colouration as a defence mechanism works especially well when tadpoles are in aggregations and this is facilitated by kin recognition (Waldman & Adler, 1979). This is because predators are more likely to see a group of animals with the same aposematic colouration rather then just one individual alone.

Xenopus laevis, otherwise known as the South African clawed frog, has very poor aquatic vision, with their eyes situated on top of their head (Elepfandt, 1996). *Xenopus laevis* frogs vocalise, with the males' calling being quite common while females call during the breeding season (Tobias et al., 2004; Tobias et al., 1998).

Xenopus is closely related to the Surinam toad which has been shown to communicate chemically (Rabb & Rabb, 1963) therefore I predicted that *Xenopus* communicate similarly. *Xenopus* live in murky waters and have poor vision. These factors suggest that they rely strongly on chemical communication. Here I investigate whether *Xenopus* do in fact communicate chemically, at the different life stages, adult and tadpole.

2.2 Initial testing of adults

Methods

I initially tested both adult males and adult females of *Xenopus laevis* for chemical communication.

Subject animals were isolated in 14 L of aged tap water and were tested over 12 nights. The experiment started with an initial control night. This was followed by a treatment night, and this sequence was repeated over 12 nights. For the control-night tests, I put 14 L of aged tap water into the tank. For experimental groups, I put in 14 L of aged tap water that had held a treatment frog for 24 hours. There were six different treatment frogs (three different females and three different males). Male treatment water was made by conditioning in the following way: one male was placed in 14 L tank of aged tap water and left for 24 hrs, before being returned to its home tank. Treatment water was taken to the experimental room where the subject frog was placed in it. A camera (Sony digital handycam DCR – TRV 19E) recorded the frog's movement and a hydrophone (High tech HTI-96-min series) recorded the noise

made by the frog. Recording lasted one hr, when the subject was removed and the treatment tank cleaned. The tanks were scrubbed with ethanol and thoroughly washed out with tap water and left to drain.

The video recording was changed to 15x speed on a VHS video tape and analysed on Noldus Ethovision. Subjects were tracked and recordings made. These recordings included the distance (cm) travelled by the frog between two samples, the maximum distance moved (cm), "maximum" being the largest value and movement 'determined by comparing objects velocity with user-defined threshold'; mean speed (cm/s), defined as the distance moved per unit of time; total movement duration (s); percentage of total movement duration (%); semi-vertical position frequency and semi-vertical position duration (s) (the semi-vertical position was a behaviour that I had previously observed and it involved the body of the subject being held at more than around 75° angle in a vertical direction). Recordings of all but one of these behaviours were automated by the computer program. Semi-vertical positioning data, was recorded manually in Ethovision at the same time as the computer read the tape.

Initial results

For the different variables tested (e.g. speed, distance moved) across the different treatment groups (control, male and female), males were considerably different from the females. Although males were generally more active throughout the experiment, the females showed a more obvious trend between treatment groups (see Figures 1, 3, 5). Female subjects were more active during female treatment testing than during either control or male treatment testing (see Figures 1, 3, 5). The increase in activity was most evident with the following variables: distance moved, mean speed and semi-vertical frequency, with this response being

more obvious in female subjects than with male subjects. I subsequently focused only on female subjects with female treatments and controls. (see Figures 1-6).

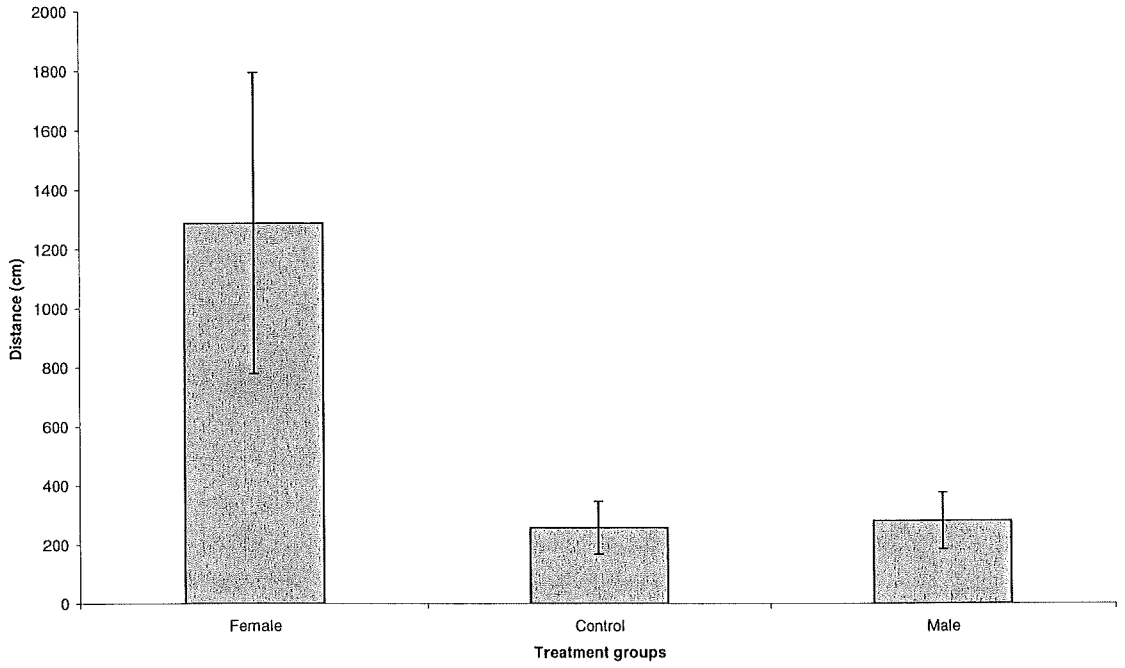

Figure 1 Mean (with standard error bars) of the total distance moved (cm) by the three female subjects from different treatment groups

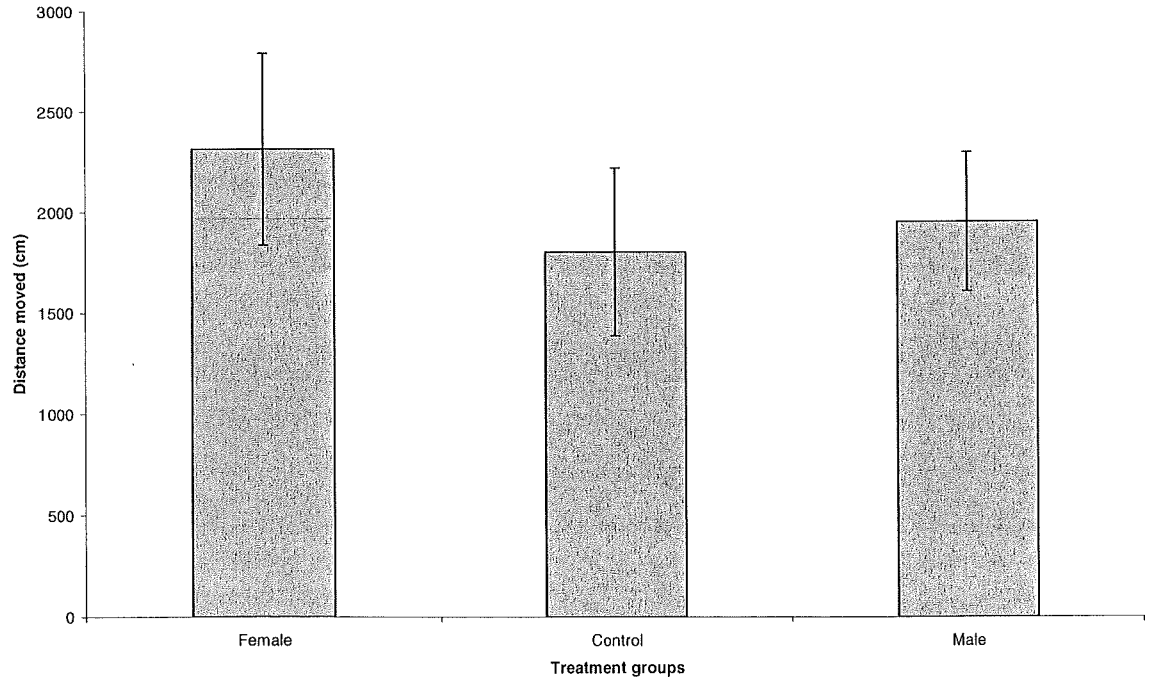

Figure 2 Mean (with standard error bars) of the total distance moved (cm) by the three male subjects from different treatment groups

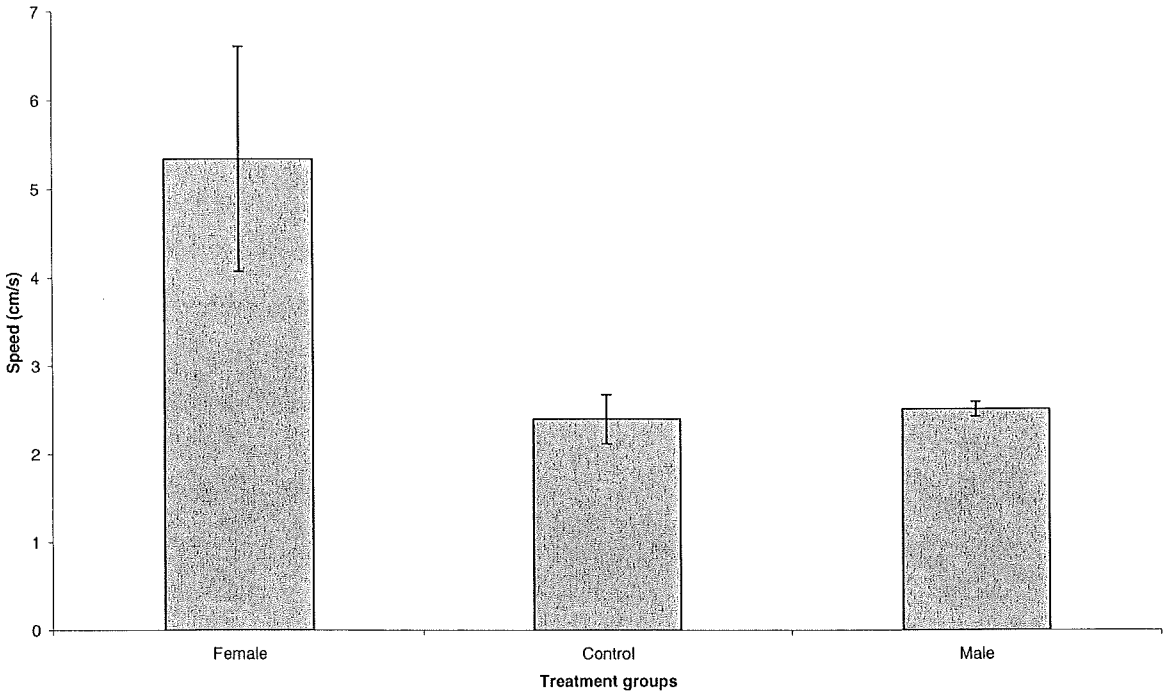

Figure 3 Mean speed (cm/s) (with standard error bars) moved by the three female subjects from different the treatments groups

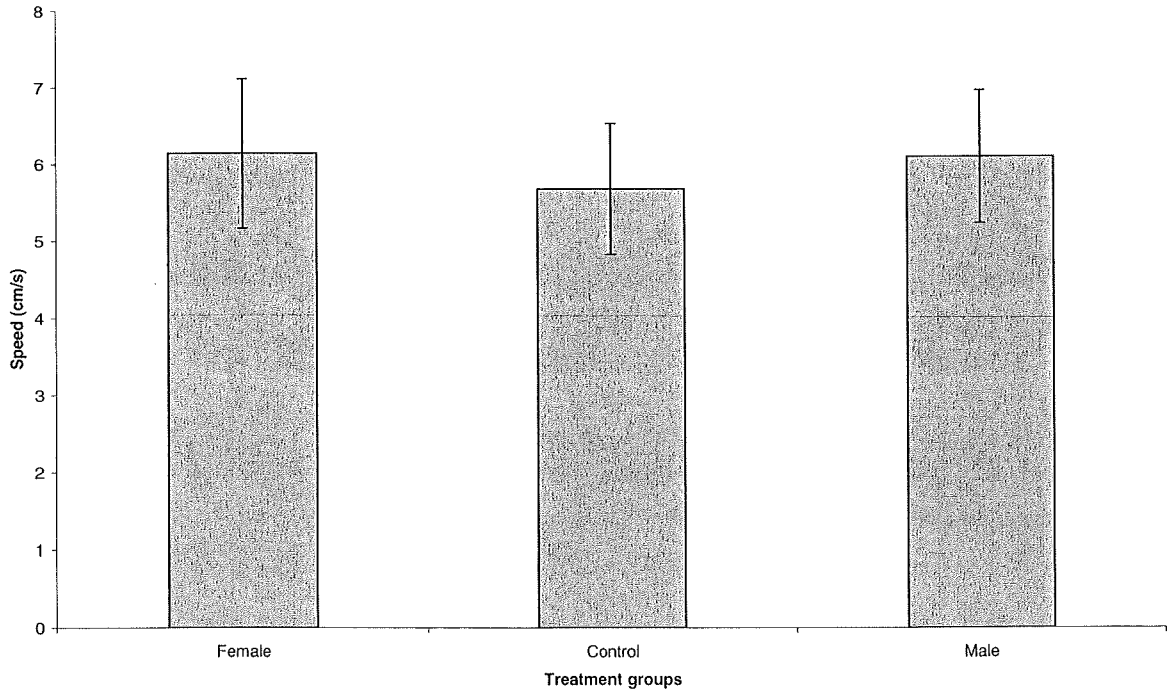

Figure 4 Mean speed (cm/s) (with standard error bars) moved by the three male subjects from different the treatments groups

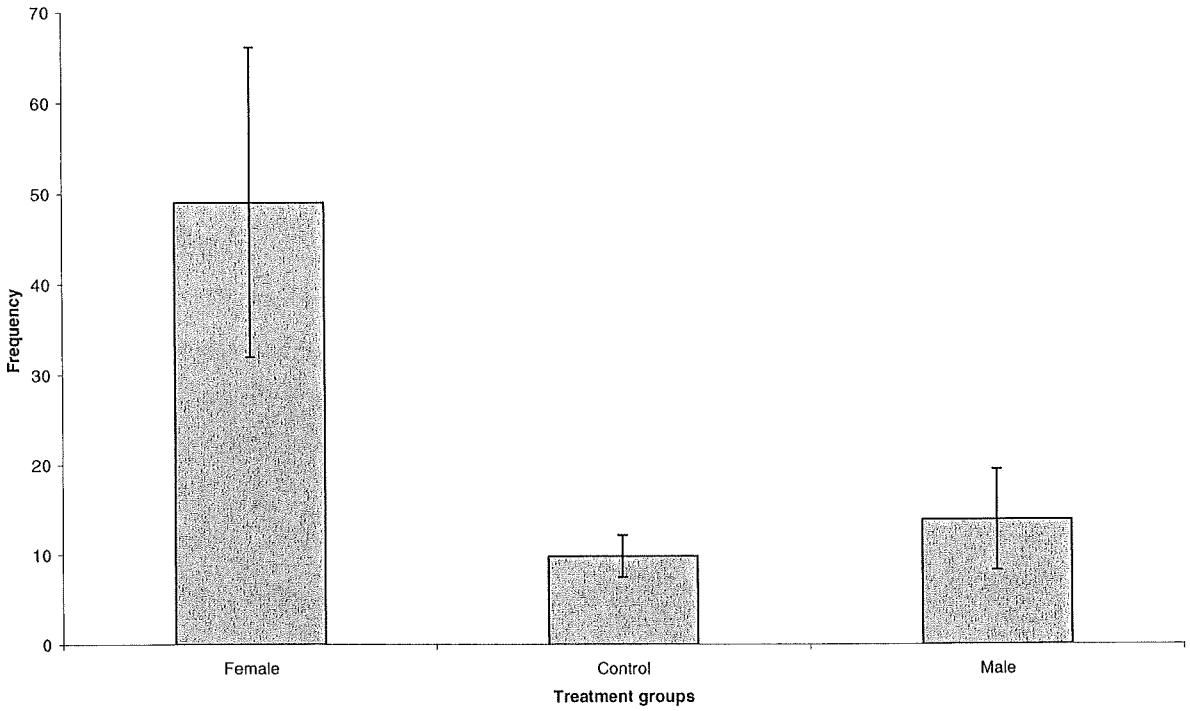

Figure 5 Mean frequency of behaviour repetitions (with standard error bars) by the three female subjects from different treatments groups

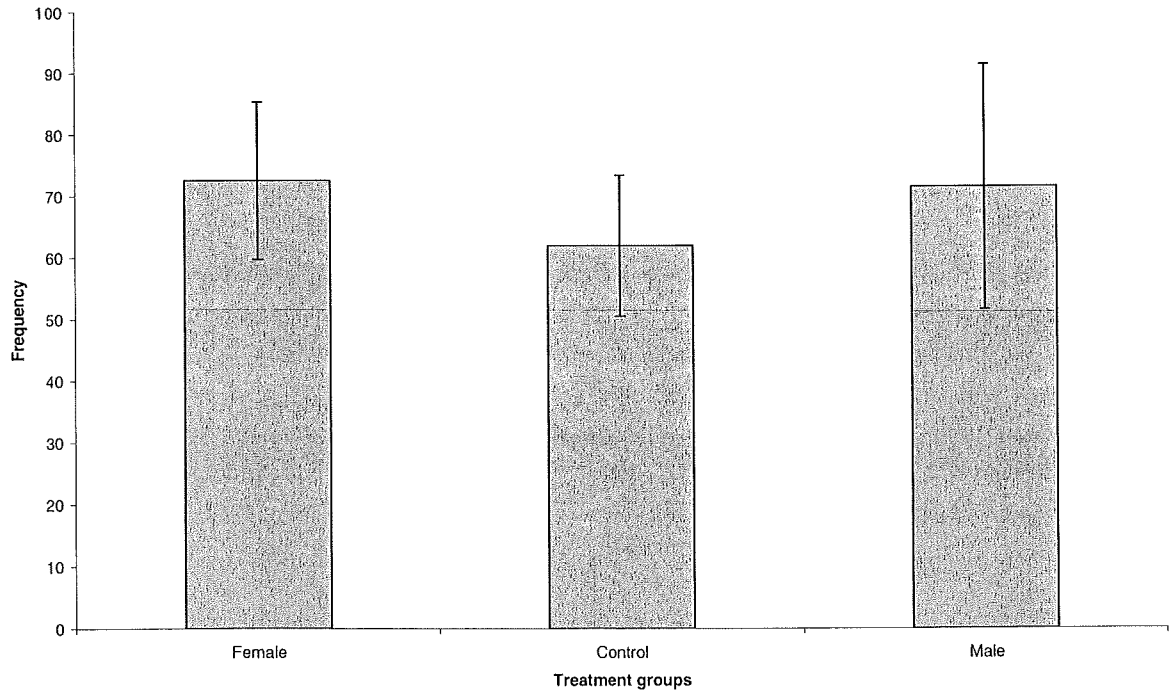

Figure 6 Mean frequency of behaviour repetition (with standard error bars) by the three male subjects from different treatments groups

2.3 Second set of tests of adults

Methods

These experiments were conducted under the same conditions as the initial experiments except that males were excluded and hydrophones were not used; females showed no vocalisation during the previous tests. Therefore, experimental runs were only six nights per subject. A further 10 female subjects were tested under these conditions.

2.4 Results

Female subjects were more active in female treatment water than in control water. Distance and speed moved was significantly increased and the there was an increase in repetitions of the semi-vertical behaviour when females were placed in female treatment water relative to control water (Figure 7-9).

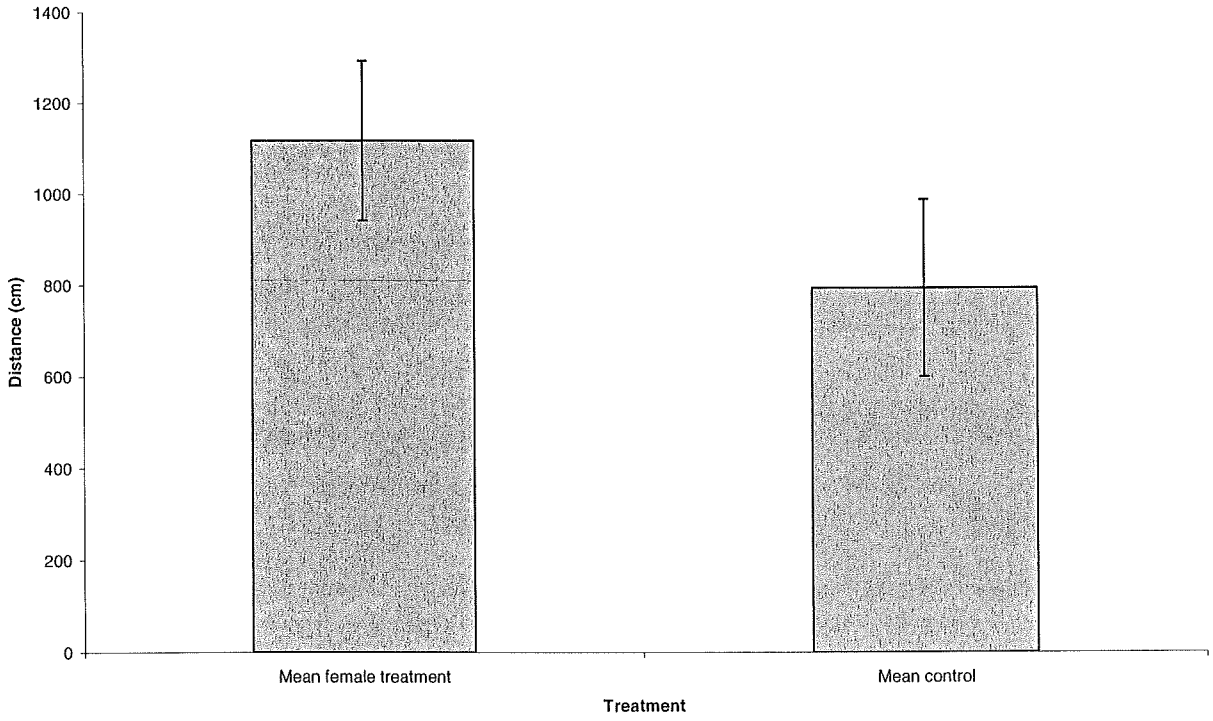

Figure 7 Mean (with standard error bars) distance moved by all of the female subjects in the two different treatment groups (N=10)

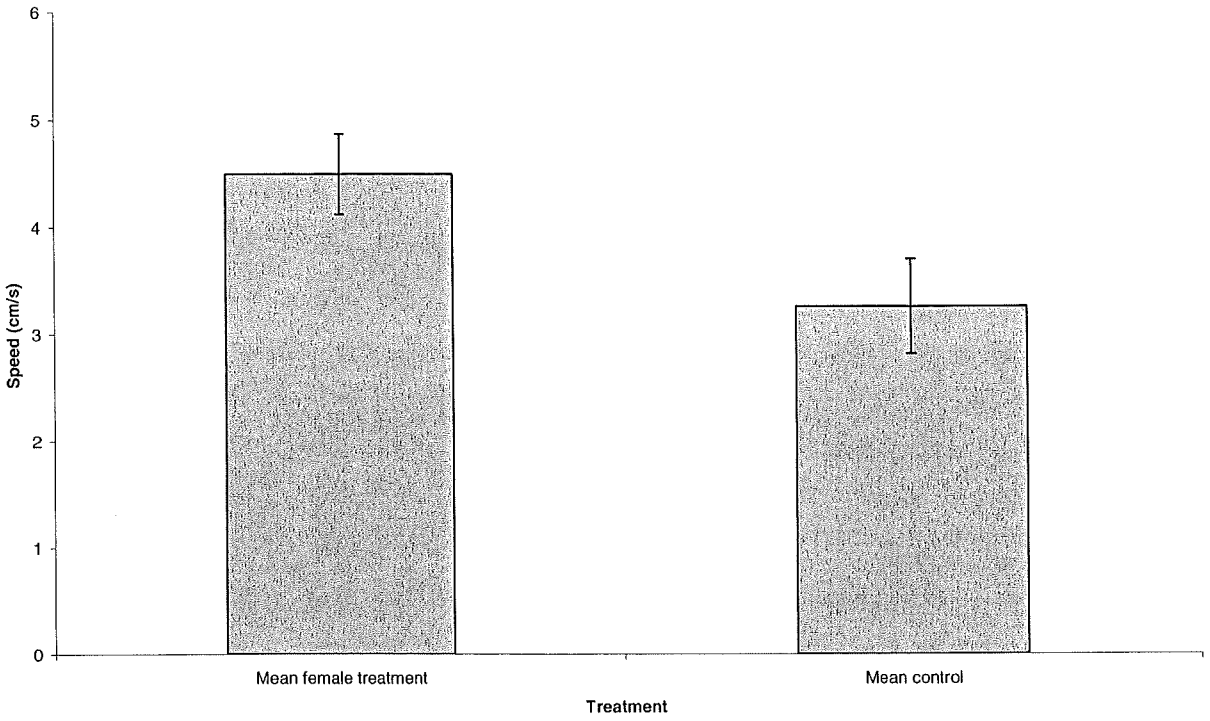

Figure 8 Mean speed moved (with standard error bars) by all of the female subjects in the two different treatment groups (N=10)

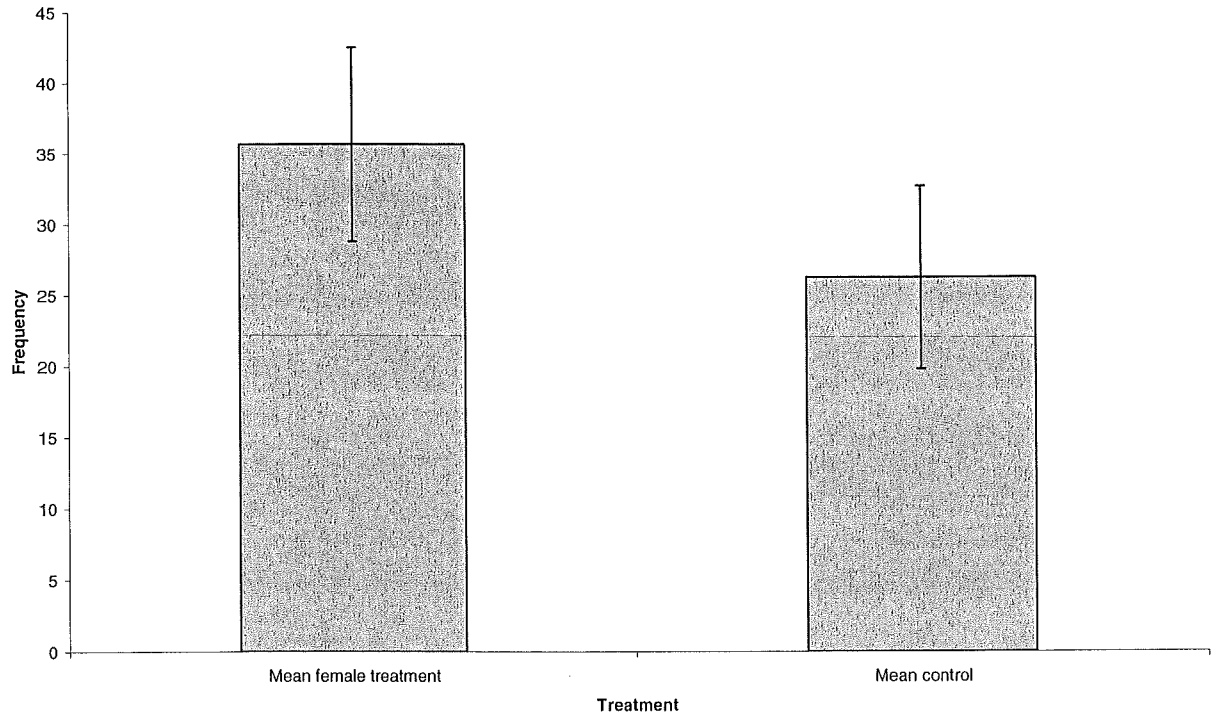

Figure 9 Mean frequency of a behaviour repetition (with standard error bars) by all of the female subjects in the two different treatment groups (N=10)

The results for the different behavioural responses (e.g. distance moved and speed) were not normally distributed. Therefore, a Wilcoxon Signed Rank Test (one tailed) was used to discern whether there was any significant difference between the behaviour of the female subject when in control versus female-treatment water. I had previously hypothesised that there would be an increase in movement when the subject was in the treatment water, therefore one-tailed tests were used. There was a significant difference between treatments for total distance moved (N= 13, W= 71.0, P= 0.040 (MINITAB 14)), and for speed of movement between the two treatments (N= 13, W= 82.0, P= 0.006 (MINITAB 14)). Differences in repetition of semi-vertical behaviour were not significant between the control and female treatment nights. (Wilcoxon Signed Rank Test N= 13, W= 60.5, P= 0.155).

2.5 Testing of tadpoles

Methods

Five different families of outbred and inbred frogs (outbred frogs originating from the wild and inbred frogs derived from laboratory line) were bred using lutenizing hormone releasing hormone (LHRH) (Argent Laboratory, 8702, 1-52nd Ave, North East, Redmond, WA, 98052 USA) to produce a sufficient number of tadpoles. Once the tadpoles were more than 6-7 days old (i.e., once they had developed enough to be free swimming and to no longer be clinging to the sides of the container), they were separated within families to reduce overcrowding and to increase rate of maturation and fed ground nettle tea ad lib to sustain growth rate. They were held in a temperature-controlled room at 22°C with a photoperiod of 12 hours light and 12 hours dark (0800 – 2000 light). The tadpoles were ready for experimentation at stages 48-50 (see Nieuwkoop and Faber 1994),

The experimental test tank contained two removable mesh nets, one at either end of the tank. At one end of the tank, the net held 10 of the subject's kin. At the other end of the tank, the net held 10 of the subject's nonkin. Both were identical in size. Mesh netting was attached to the sides of the middle compartment (where the subject was held) to ensure that all of the sides were similar in appearance. A line was drawn down the middle of the tank creating two halves.

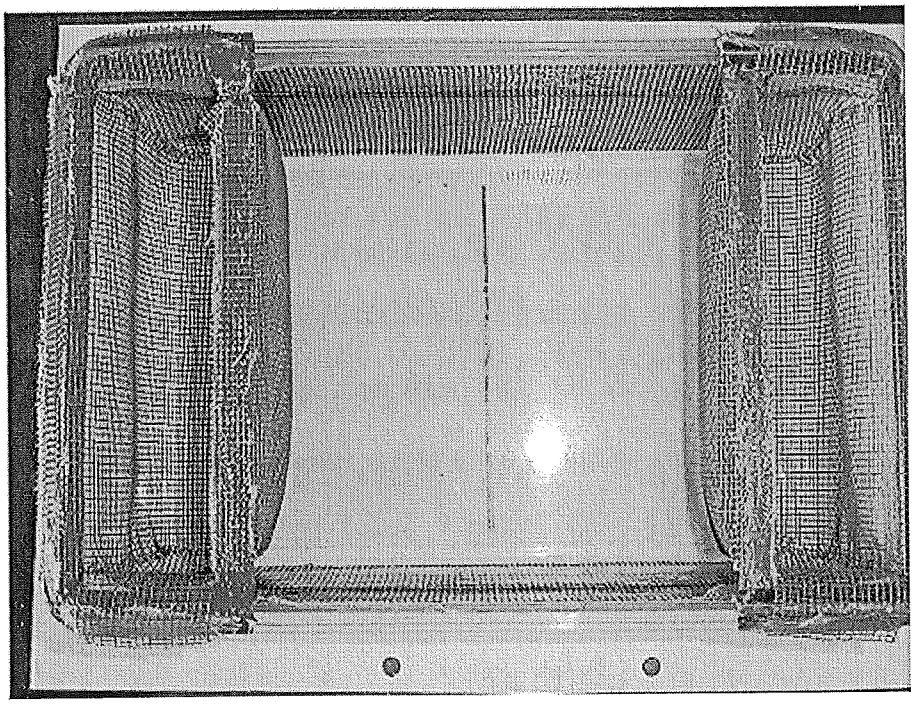

Figure 10 Photograph of the tadpole kin-preference experiment tank

One hour recordings were made to observe any potential side bias.

Side-bias tests were conducted in the experimental room to evaluate the quality of external noise. These tests involved all six tanks. Three tanks had the subject's kin on both ends of the tank and the other three tanks have empty nets at each end. The tanks were arranged in a checker board pattern. The location of the tanks under the camera was alternated with a new subject each time. The test was repeated for two families with 30 subjects in each. There was no significant side bias in the room.

Six tanks were run simultaneously with their positions randomised within a grid under the camera (two rows and three columns). The tanks were further divided into groups A and B, with tanks 1-3 in group "A" and 4-6 in group "B". "A" tanks always had the subjects' kin on the right side of the tank, and "B" tanks always had the subjects' kin on the left side of the tanks. This randomisation compensated for any side bias that could have occurred during the experiments.

Tests were conducted in a dark room to avoid potential influences of light changes throughout the day. There were 30 replicates (subjects) within each of the five families. Each experimental run was for 1 hr, after a 5 minute acclimation period.

The video was recorded directly at 15x speed on a VHS tape and then run through Noldus Ethovision. The length of time spent on each side of the tank and the duration of movement on each side was recorded.

This method was repeated for both outbred and inbred frogs.

2.6 Results

First I will consider the results from kin recognition tests for outbred tadpoles using a two-way choice tank for a period of 60 min, after a 5 min acclimation period. These results were variable. When analysing the mean time spent on kin side for each family, there was no significant difference (Wilcoxon signed- rank test (MINITAB 14) (see Table 1.)). A paired t-test and a Wilcoxon signed-rank test were calculated, using the difference between the amount of time (s) spent on kin side for all individuals per family with the amount of time (s) spent on nonkin side for all individuals per family (i.e. kin – nonkin). There appeared to be a trend, as three out of the five families spent more time on the kin side of the tank (Table 1.).

However, when counting, for each family, the number of individuals within each family that preferred to spend more time on kinside, there were only two out of five families that showed kin preference (Figure 11.). The data were taken as random when half of the individuals preferred kin side and the other half preferred the nonkin side.

With this trend in mind, I decided to investigate whether the trend was amplified when using inbred frogs with homozygous alleles. The rationale for this was from the results by a fellow student (J. Villinger unpublished). The experiments were carried out under the same conditions as the outbred-families experiments. The results from the inbred families were very similar to the results from outbred families, with three families showing a bias towards selecting kin, but some of these trends were only slight (Table 1.). One of the inbred families had a significant P value, but in this instance it was showing a preference for nonkin. Two out of five families spent more time on the kin side. Family FF was random, having a score of 15 out of 30 individuals preferring kin (Figure 12.).

The experiments were again modified to see whether the trends would be stronger when all of the tadpoles involved in the experiment were isolated for 2 weeks before the experimental period. Part of the logic behind isolating the tadpoles was that isolation would partially stress the tadpoles, possibly resulting in them responding more strongly. In addition, I reduced the amount of food they were fed, the rationale being to control for the possible overpowering smell of food. The results from this isolation experiment also showed, three families out of five preferred kin (Table 1). When examining the number of individuals within a family that preferred kin, there are only two families, FF and JJb, that showed kin preference (Figure 13).

Table 1 Mean time spent on kin and nonkin side of the test tank during the kin recognition experiments, by three different groups, outbred, inbred and inbred isolated tadpoles. N= number of individuals, T= Paired T-test value, df= degrees of freedom, P <0.05 probability, P 37= Wilcoxon Signed Rank Test at 0.05, W= Wilcoxon statistic. (MINITAB 14). The Paired T-test and Wilcoxon signed rank test were calculated using the difference between the amount of time (s) spent on kin side for all individuals per family with the amount of time (s) spent on nonkin side for all individual per family. Medians for isolated inbred families for both kin and nonkin side preference.

		Mean time spent (s)		Standard error					
		Kin side	Nonkin side	Kin	Nonkin	N	T	df	P
Outbred Family	F1	340.5	319.5	17.5	17.5	30	0.60	29	0.55
	F2	361.6	298.4	29.5	29.5	30	1.07	29	0.29
	F3	329.6	330.4	23.8	23.8	30			
	F4	308.0	352.0	29.4	29.4	30	-0.75	29	0.46
	F5	349.1	310.9	13.1	13.1	30	1.46	29	0.15
Inbred Family	FF	330.5	329.5	20.4	20.4	30	0.03	29	0.98
	JJ	349.3	310.7	21.8	21.8	30	0.88	29	0.38
	GG	301.8	358.2	20.5	20.5	30	-1.38	29	0.18
	FFb	331.0	329.0	29.4	29.4	30	0.03	29	0.97
	JJb	305.0	355.0	13.4	13.4	30	-1.87	29	0.07
						N	W	P	
Inbred - isolated Family	FF	343.7	316.3	21.1	21.1	30	280	0.33	
	JJ	298.8	361.2	16.4	16.4	30	167	0.18	
	GG	330.6	329.4	16.5	16.5	27	159	0.48	
	FFb	326.3	333.7	11.6	11.6	27	183	0.90	
	JJb	356.1	303.9	23.2	23.2	28	244	0.36	

	Medians for Isolated inbred tadpoles	
	Kin side	Nonkin side
FF	336.6	323.4
JJ	323.3	336.7
GG	317.2	342.8
FFb	328.8	331.2
JJb	339.3	320.7

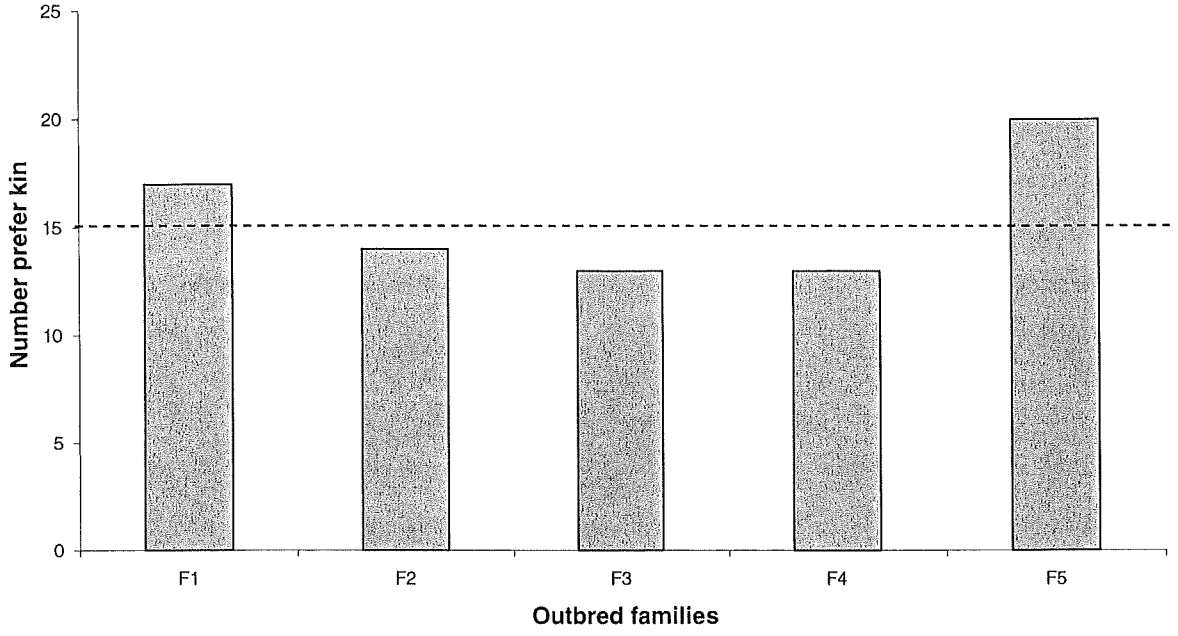

Figure 11 Number of outbred tadpoles that prefer kin. N= 30 for all families (dotted line is probability of random)

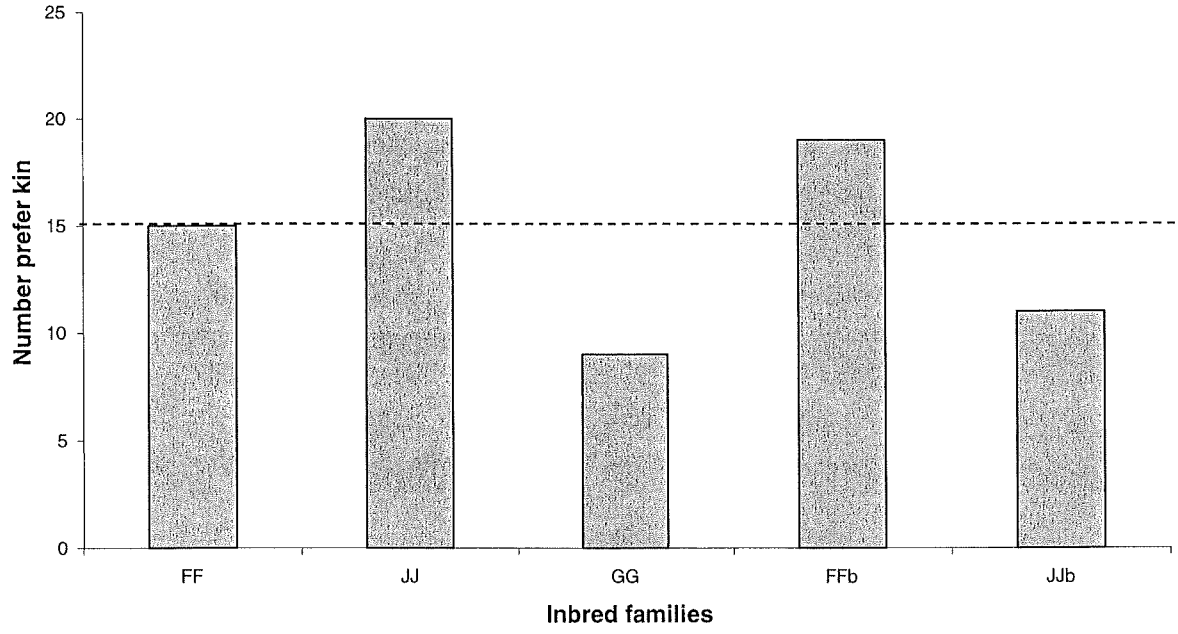

Figure 12 Number of inbred tadpoles that prefer kin. N= 30 for all families. (dotted line is probability of random)

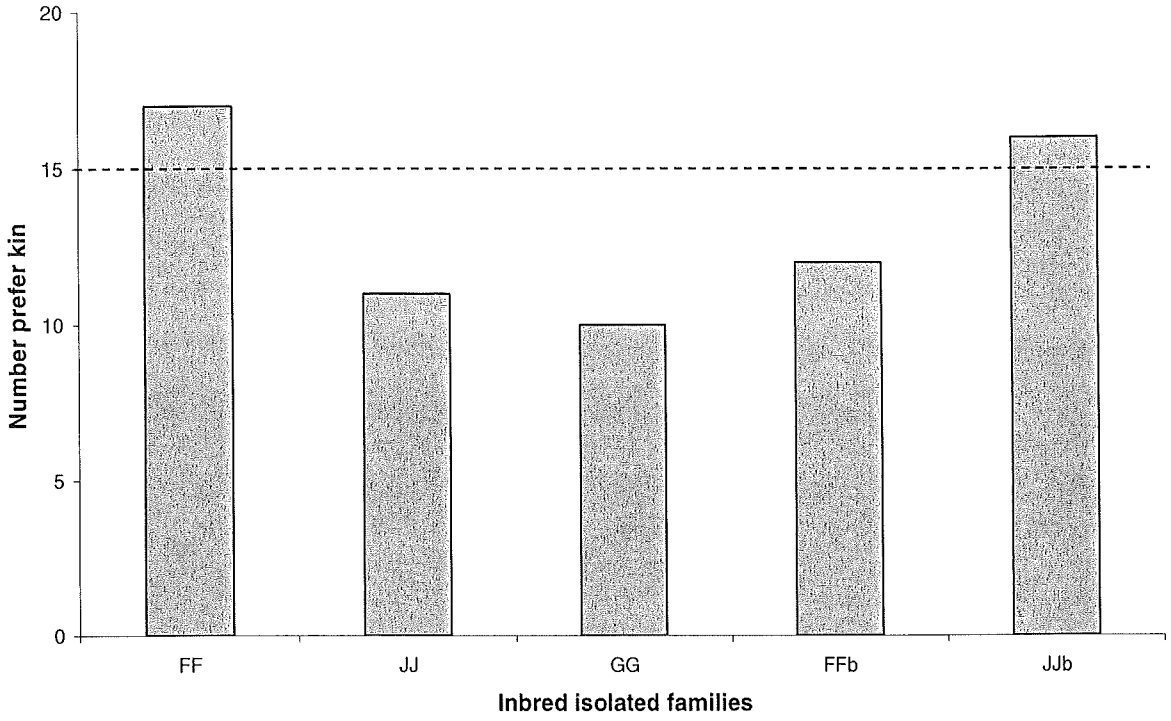

Figure 13 Number of isolated inbred tadpoles that prefer kin. N= 30 for families FF and JJ, N= 27 for families GG and FFb, and N= 28 for family JJb. (dotted line is probability of random)

2.7 Discussion

Amphibians have four different modalities of communication, vocal (Hodl & Amezquita, 2001; Doherty, 1994; Waldman, 2001), visual (Hodl & Amezquita, 2001), seismic (Narins, 1990; Narins, 1995) and chemical (Lee & Waldman, 2002; Waldman & Bishop, 2003). I have investigated whether individuals of *Xenopus laevis* chemically communicate with one another. Chemical communication can be used to convey, fitness (Mathis, 1991), to indicate territories (Horne & Jaegar, 1988), in courtship (Horne & Jaegar, 1988), for recognition of kin (Waldman, 1981), for alarm signals (Hews, 1988; Hews & Blaustein, 1985) and as aids during homing (Grubb, 1973; Madison, 1969). The rationale for predicting that *X. laevis* adults chemically communicate included, the murky waters in which they live (Tinsley et al., 1996), their poor vision and the dorsal positioning of their eyes for aerial predators (Elepfandt, 1996). Further, a related species, the Surinam toad, can chemically communicate

(Rabb & Rabb, 1963). I also theorised that tadpoles would also chemically communicate, and I tested chemical communication in the context of recognition because several species have been shown to recognise kin (Waldman, 1981; Waldman, 1985; Waldman & Adler, 1979; Blaustein & O'Hara, 1982; Blaustein & Waldman, 1992), and there may be strong benefits for individuals capable of recognising kin (See Chapter 1).

My experimental results suggested that *Xenopus laevis* adult frogs communicate chemically. During initial experiments, in which I tested both adult male and adult female frogs, there was a statistically different behavioural response by the females between control and experimental nights. Males did not show any significant difference between the different treatment groups (control and experimental nights). Variables that showed the most significant differences between control and experimental nights were distance moved, mean speed and semi-vertical frequency. Female subjects showed a significant increase in activity (for all three of the variables) when tested in female treatment water relative to control and compared with testing with the male treatment water. Because the behavioural response of female subjects in female water was so strong, I decided to concentrate the next phase of my work on the female subject in female water assay, for which I got the most promising results in the initial study. A further 10 females, tested in this manner, also showed an increase in activity for all three variables when exposed to water that had previously held another female, in comparison to their activity in the control water. There was a significant difference between the control nights and female water for two of the three variables (speed and distance moved) but not for the variable semi-vertical behaviour frequency. Evidently, female subjects can discriminate between control water and water that has held another female frog. An alternative explanation for theses results might be suggested that the behavioural response is accounted for by the water being 'dirty' (i.e. from previously housing a different frog). Had this been the case, the

female subjects would have been responding the same way for male and female dirty water, and also the male subjects would have responded similarly. This however was not the case.

Unexpectedly I detected a differential response to the assay between sexes. I initially hypothesized that because of possible sex pheromones the male subjects would be more responsive to the female water, and that territoriality would make male water the next most effective treatment. The weak response by the male subject towards the female water was surprising in comparison to previous work on the Surinam toad showing female water showed male excitation and calling (Rabb & Rabb, 1963). This weak of response by *Xenopus laevis* male subjects may be explained because there was no secondary cue. For example, males may normally get excited by the female water but not be inclined to continue pursuing this behaviour when there is no physical presence of the female (cf. (Rabb & Rabb, 1963)), or the male subject may be aware of the male water but not behave territorially because again there is no physical presence.

My results contrasted with Rabb and Rabb (1963). What I found was that the females showed the strongest response to female treatment water out of all of the tests. The increase in activity when exposed to female treatment water suggests that, in this species of frog it is the female that is especially territorial. Male *Xenopus laevis* may be more inclined than females to leave the natal site and therefore have no defined home range so their defensive behaviours occur only when proximate to another frog. By contrast, females possibly do not migrate as far and are therefore likely to be in the presence of other frogs; when an unfamiliar frog is 'sensed' they become especially territorial. This hypothesis could fall under Fisher's theory called 'Dear enemy' (1954), in which an animal will become more aggressive when receiving cues from a stranger rather than a known neighbour because once the territories have been

established neighbours pose little threat. This has been shown in red-backed salamanders (*Plethodon cinereus*) (Jaegar, 1984; Jaegar, 1981). Further, red-backed salamander females are more responsive to faecal pellets than males, possibly because exclusive occupation of territory security is especially important for the survival and reproductive success of the female (Horne & Jaegar, 1988). This could explain the behaviour of *X. laevis* frogs in this experiment.

The lack of behavioural change in the subject females with exposure to male treatment water could be due to the release of a courtship pheromone after the initial courtship interaction has occurred, a phenomenon observed in many amphibians (Houck et al., 1998). If this is true for *X. laevis,* then this may explain why the female did not behave differently between the control and male treatment tests.

The results of the adult chemical communication experiments do not provide unequivocal evidence that *Xenopus laevis* individuals communicate chemically. Further studies need to be carried out to examine more thoroughly how and when they chemically communicate. The assay may need to be modified to more effectively reveal the importance of chemical communication. Future studies should perhaps be based on using animals that the female subjects 'know' and compare those results with results from testing female subject with animals they do not 'know'. These results could ascertain the validity of the 'Dear enemy' hypothesis.

Tadpole testing results differed from those of the adults tests. Kin recognition was considered when testing the tadpoles for chemically communication and kin recognition has been shown to occur in many species of tadpoles (Waldman, 1981; Waldman, 1985; Waldman, 1986; Blaustein & O'Hara, 1982; Blaustein & Waldman, 1992).

The initial tadpoles tested were from five different outbred populations (within the laboratory). Three of the five outbred families spent more time on the kin side of the tank, but this trend was not statistically significant. Within the family, the number of tadpoles that spent more time on the kin side was only two out of three. A slight trend was suggested for kin preference when the mean time spent on kin side was examined but it was not as powerful as anticipated. It was for this reason that I repeated the tests, but now using five inbred families. It was thought that with MHC homozygous genes, the trend would be more evident (J. Villinger personal communication). The inbred experiments were repeated in the same manner as the outbred experiments. These experiments showed the same trend as for the outbred tadpoles. One family showed a significant result but it was for spending more time on the nonkin side. Finally I isolated all of the tadpoles (subjects and net tadpoles) in order to stress them, and therefore strengthen the response. In addition, tadpoles were fed less to make sure that subjects were not responding simply to the smell of food coming from the tadpoles in the nets. The results from these experiments were the same; three out of five spending their time on kin side and only two out of five spending more time on the kin side.

My results suggest, regardless of whether the tadpole is of outbred or inbred origin there possibly is kin recognition. A previous study using *X. laevis* by M. Locker (1989) (unpublished) showed a strong kin preference. Locker studied inbred tadpoles but only recorded the initial ten minutes. In addition, Locker had three partitions with the middle partitions being a neutral zone. I reanalysed my results according to Lockers methods and found that the data was rather random with time spent on kin side being very similar to the time spent on nonkin side. When analysing three divisions for the full time, again there was not much variation in results, this was the same with three divisions for ten minutes. I decided

to stick to the original method of analysing the data because it gave the most robust data and seemed the most logical.

Several reasons may explain why my data for kin recognition were not as strong as has been shown in previous studies on other species of tadpoles. The most likely explanation is variation in the methodology, for example, the size of the tank. Because, in behaviour tests even slight changes in the state of the variables can make the difference between significant results and negative results. For future work, modifications include; size of the tank changing, number of tadpoles in each of the nets, the age of the tadpoles, the time of day when the tests are done, starvation before testing, heritage of the subjects, period of experimentation, temperature, and external noises.

Chemical communication occurs in adult female *Xenopus laevis*, and in the tadpole tests there was an encouraging trend.

More studies could be conducted, the experimental tank could be modified so the subject can not see the tadpoles held in the nets, but making sure that chemicals can still pass through, thereby ruling out confounding influences of cues based on vision. Testing metamorphosed frogs for chemical communication would be interesting to see if chemical communication is needed in all of the life stages. Experiments on adults could be refined to examine whether the male frogs do communicate chemically and if so under what conditions. A tank could be arranged so the subject frog can see through a barrier to another frog and observe whether the male response to the treatment water is greater when it can see the other frog.

Chemical communication is an important component to study. Once baseline data are established, many variables can be modified to examine possible external impacts on the

communication system, for example toxicants. Extension of this study, to evaluate toxicants, could help with the understanding one of the reasons for amphibian decline.

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Chapter 3

The Effects of Exposure to Atrazine

3.1 Introduction

The application of pesticides, herbicides and insecticides that we deposit onto our environment, whether by application, spillage or runoff, may have a large unintentional impact on other parts of the fauna and flora. Besides the obvious impacts that we can measure (e.g. death and the decline of species), there may also be sublethal effects, which may not be identified until it is too late. These effects can impact on food webs, mating success and ultimately survivorship. Sublethal effects of toxicants may affect the animal's communication system. Small amounts of toxicants that do not actually kill the animal can render the animal impaired in some way or another. These effects can be subtle, acting on the endocrine system or other internal functions of the animal. Because these kinds of impairments may not be 'seen' until the animal is fully mature, an animal may seem to be physically fine when it is not.

Endosulfan is an insecticide that has negative impacts on the environment and has been banned in several countries. In particular, it has been shown to impair the pheromonal communication system of the red-spotted newt, *Notopthalmus viridescens*. At sublethal levels, endosulfan (5 to 10 ppb) exposure directly impacts the mating success of these animals by disrupting mate choice through olfactory communication and causing physiological changes that decrease the size of the alveoli in male newts, even when there were no signs of any behavioural changes (Park et al., 2001; Park & Propper, 2002). The effects of endosulfan on the growth of the alveoli suggests that this insecticide disrupts the excretion of steroid hormones and prolactin (Park & Propper, 2002).

Atrazine (2-chloro-4ethylamino-6isopropylamino-*s*-triazine) is a triazine herbicide that is effective specifically in the control of broadleaf and some grassy weeds. It kills the selected plant by inhibiting photosynthesis (EPA., 2001; Solomon *et al.*, 1996) This white crystalline organic compound is often mixed with liquid (Services., 2003) and used on corn, maize, sorghum, sugarcane, landscape vegetation and maize (Solomon K.R *et al.*, 1996; Hang *et al.*, 2003; Lakshimnarayan *et al.*, 1996; Wolf SA & Nowak PJ, 1996).

Atrazine was first used in Switzerland in 1958 as an unregistered herbicide. Then, in 1959, it was used commercially in the United States. Since 1959, atrazine has been the major herbicide used worldwide (Solomon K.R *et al.*, 1996). In the United States in 1993 approximate 35,000 tons of atrazine was used, and in the Midwest area of the United States in 1995, it is estimated that 53 million pounds of the active ingredient was used (Sanderson *et al.*, 2001; Lakshimnarayan *et al.*, 1996).

In 1994 the United States Environmental Protection Agency (EPA) initiated a review of atrazine because of alarms about residue. The EPA concluded that atrazine had "the potential to affect aquatic organisms and terrestrial plants and their ecosystems" (Solomon K.R *et al.*, 1996). In 1991 the Wisconsin Department of Agriculture, Trade and Consumer Protection (WDATCP) set up field-application restrictions in addition to the federal standard for use of atrazine. It reinforced a preventative action limit of 0.35 ppb, and the application of atrazine was restricted between the period of April 15th and July 31st. In 1993 the total amount of land that had a prohibition of atrazine in the Wisconsin region was 1,000,000 acres (Wolf SA & Nowak PJ, 1996).

The United Sates EPA specifies a maximum contaminant level (MCL) for toxicants in drinking water. Atrazine has a MCL of $3\mu g/L^{-1}$ (Shipitalo & Owens, 2003)

Although the effects that atrazine can have on humans are still being studied, atrazine is listed by the EPA as a class C compound in many parts of the United States, and is designated a possible carcinogen for humans (Wolf & Nowak, 1996). Evidence for this conclusion came especially from Adalberto *et al.* (1989) who showed that women who had been exposed to triazine herbicides had a two to threefold increase in the risk of epithelial ovarian cancer compared to women who had not been exposed.

The impact of atrazine on rats has been investigated and attempts have been made to relate information from these studies back to humans. For example, the rat's lateral and dorsal prostate are analogous to the human prostate, and is also responsive to prolactin (Stoker *et al.*, 1999). With this knowledge, Stoker *et al* (1999) exposed postnatal rats in the first four days of lactation to atrazine and found a suppression of maternal suckling-induced prolactin release and resulting in secondary effect on the adult offspring by increasing the frequency of inflammation of the lateral prostate. When rats were exposed to atrazine (12.5 to 200 mg/kg), reproductive-tract development and pubertal progression of the male was delayed (Stoker et al., 1999). Stoker *et al.* (1999) interpreted these findings as evidence that atrazine disrupts the central nervous system's control of pituitary function (Stoker *et al.*, 1999). Babic-Gojmerac *et al.* (1989) showed that atrazine significantly increases the wet weight of the pituitary glands. Testosterone levels in male rats are also affected by atrazine exposure. At an exposure of 50 mg/kg of atrazine, male rats experienced about a 50% decrease in intratesticular testosterone and serum. Atrazine may cause this decrease in testosterone by inhibiting testosterone production (Friedmann, 2002). Findings from studies conducted on female rats have been similar, showing that as little as 50 mg/kg atrazine can change the estrous cycle and delay the

onset of puberty (Laws *et al.*, 2000). Atrazine can also induce mammary tumours, uterine carcinomas and leukemias/lymphomas in male and female rats. These results which were seen at exposure rates between 375 and 750 ppm atrazine, were dose-dependent (Pinter *et al.*, 1990).

The half-life of atrazine can vary depending on ecological factors, generally ranging between 8 and 350 days (Tavera-Mendoza. *et al.*, 2002a). Through agricultural runoff or by direct careless application, atrazine can enter waterways such as streams and ponds (Hussein. *et al.*, 1996). Atrazine can alter the chemistry of water by causing a decrease in the pH, dissolved oxygen and so available food (Hayes et al., 2002a).

After storm runoffs atrazine can be found in concentrations of 20 μg/L or more in some streams, but these are only transient pulses (Solomon. *et al.*, 1996). Atrazine can affect amphibians by reducing swimming speed and causing lethargic behaviour (Sparling et al., 2000; Howe et al., 1998).

The high levels of atrazine found in many streams and ponds have raised concern for how contaminated water may affect living animals, which are continuously exposed to such herbicides. In two fish species, *Oreochromis niloticus* and *Chrysichthyes auratus,* atrazine increases gill cover movement and respiration rate, as well as swimming movement and reflexes, with feeding activities also being reduced. Atrazine also decreases the haematocrit, hemoglobin and the red number of blood cells (Hussein et al 1996).

Amphibians are at high risk from exposure to toxicants due to their biphasic lifestyle, either while in the larval stage or in the adult stage (Stebbins & Cohen, 1995; Wilbur et al., 1990),

and this is one of the reasons why amphibians are often used as a bioindicator of environmental health (Stebbins & Cohen, 1995).

Hayes *et al.* (2002) studied the impact of ecologically relevant levels of atrazine on metamorphosis and sexual differentiation in African clawed frog (*Xenopus laevis).* They found that, at any atrazine level there was little effect on the mortality, growth rate, body length, body weight and age at which metamorphosis took place. However, at all the doses of atrazine tested, there were gonadal abnormalities, such as male and female gonads within a single individual (i.e. hermaphrodism). In addition, the larynge size of male frogs was reduced and the plasma testosterone levels were 10 fold less. Hayes *et al.* (2002a) hypothesised that, by disrupting steriodogenesis, and possibly inhibiting testosterone and induce estrogen secretion, atrazine interferes with the endocrine system.

When looking at the effect of atrazine on the time to metamorphosis and the mass of *Xenopus laevis,* Sullivan and Spence (2003) found that, with an increase in atrazine concentration, there was a decrease in the weight of the animal and there was also an increase in the time to metamorphosis when concentration levels were increased. These findings conflicted with the findings of Hayes *et al.* (2002).

In different regions of the United States, 10-92 % of the wild leopard frogs, *Rana pipiens,* that have been sampled showed gonadal abnormalities, including hermaphroditism and retarded development (Hayes et al., 2002b). In a laboratory study, Hayes *et al.* (2002) exposed more individuals of *Rana pipiens* to different concentrations of atrazine and got results consistent with the effects of an endocrine disrupter (i.e. producing retarded development and testicular oocytes).

When exposed to 250 µg/L of atrazine, *Ambystoma tigrinum* (the tiger salamander), metamorphosed at the same rate as the controls, but were much smaller. However, when exposed to a lower dose (75 µg/L) of the atrazine, the larvae metamorphosed at a slower rate than the control, but still at a size and weight that was similar to that in the control. Larson *et al.* (1998) hypothesised that atrazine disrupts the environmental cue that triggers the production of corticosterone and thyroxine; the hormones that regulate the metamorphosis of the tiger salamander. This was interpreted as being an indirect endocrine disruptor effect.

Tavera-Mendoza *et al.* (2002a 2002b) exposed the gonadal differentiation stage of tadpoles to 21µg/L of atrazine and found that after 48 hours, a 57% decrease in the testicular volume of *Xenopus laevis* tadpoles, a 70 % decrease in the number of spermatogonial cell nests and a 74 % decrease in the number of nursing cells. There was also testicular resorption and aplasia in 70 % and 10 % respectively, of the tadpoles exposed to atrazine. Tavera-Mendoza (2002b) did a similar experiment in which female *Xenopus laevis* tadpoles in stage 56 were exposed to 21µg/L atrazine for 48 hours. After exposure, there was a decrease in the occurrence of primary oogonia, an increase in the occurrence of the secondary oogonia and an increase in the rate of atresia.

More needs to be known about the effects of atrazine because of its high level of usage across the planet (Solomon et al., 1996), and the disturbing effects that it has been proven to have on the animals that have been tested. Amphibians are in decline worldwide and the reasons why are unknown. (Stebbins & and Cohen, 1995). With atrazine being known to be an endocrine disruptor (Tavera-Mendoza. *et al.*, 2002a; Tavera-Mendoza. et al., 2002b; Larson. *et al.*, 1998; Brown Sullivan. & Spence, 2003; Hayes et al., 2002a).

I was interested in whether atrazine would interfere with the chemical communication systems of frogs. Impaired communication in turn might hinder the survival of the frogs. My hypothesis is that atrazine hinders, or blocks, the receptors for incoming chemical communication cues, alters the chemical cue itself or interferes with the transmission of the cue in the water.

If atrazine affects the endocrine system and disrupts the gender composition of a frog within 48 hrs of exposure (Tavera-Mendoza. *et al.*, 2002b; Tavera-Mendoza. *et al.*, 2002a), then it might, for example, be that androgen levels are disrupted by the atrazine and this in turn might cause changes in secondary sexual characteristics (Emerson. *et al.*, 1993). These changes might result in the courtship ritual not being properly started.

3.2 Methods

I used data from the previous chapter as my baseline data. Results in this chapter are compared to those data.

The subject tadpoles that I used were the same as for the chemical-communication tests. They were numbered for identification and reference. These initial tests were the control tests.
The experimental tanks used during the atrazine-exposure tests were the same tanks used in the chemical-communication tests. The same room and protocol were used.

Two-phase serial dilution was used for accurately obtaining a final concentration of 10 μg/L atrazine. Using an ultra-micro balance (Mettler Toledo umx2) in an eppendolf lid, 1 mg of atrazine was measured. The eppendolf lid with the atrazine on it was then placed in a flask with 1 L of distilled water (see Barnstead Nanopure D4755). The distilled water was

measured using a conical flask. The flask (with the eppendolf lid still inside) was place in a water bath (see Transtek system, Soniclean 160T 160-98-1577) and sonicated for 10 min to mix the atrazine with the distilled water until no particles could be seen. The solution was then diluted down to 10 μg/L atrazine using 5 ml pipettes to add 5 mls, of stock solution to 495 ml of aged water. This was in a conical flask. The diluted end solution was stirred using a magnetic stirrer and stirring rod (Corning Hot Plate Stirrer PC-351, with a 3 cm stirring rod) for no more than 2 min. One tadpole was added to the 500 ml container for 24 hours. This was repeated for all 30 individual within each family. The tadpoles were maintained in a temperature-controlled room at 23 °C ±1 °C, with a controlled light period of 12 hour day and 12 hour night (0800 – 2000 light). None of the subjects were fed during the exposure.

All experimental procedures were approved by University of Canterbury Animal Ethics Committee.

3.3 Results

For the inbred tadpole families the initial findings from control tests were compared to the findings from the exposure tests (10 μg/L atrazine, 24 hour period). During controls, a trend was suggested by the data. There were three out of the five families that spent more time on the kin side, but during exposure there was only one out of four families that spent more time on the kin side (Table 2). (Family four was not exposed because of extenuating circumstances.) Using paired t-tests, control scores were compared to the exposure scores for kin preference. Two of the families (F2 and F5) showed a significant difference (Table 2).

When the inbred families were exposed to atrazine, there was a similar trend. None of the families within the inbred group showed a significant preference for kin (paired t-test NS, Table 2). The results from the isolated-tadpole tests were contrary to the results from the previous tests. After exposure to atrazine, four out of the five families showed kin preference (Wilcoxon signed-rank test, NS). When analysing the data slightly differently, by looking at the number of individuals within a family that spent more time on kin side, there was no clear trend (see Fig. 14-16). For the outbred family, the results after exposure to atrazine seemed to suggest an increase in the number of individuals that preferred kin compared to a decreasing in kin preference depending on the family as a mean result. This is true for inbred families and isolated inbred families (see Fig. 14-16).

Table 2 The mean time spent (s) on kin and non kin side during a one hour experiment (after five minutes acclimation period) by three different groups, outbred, inbred and inbred isolated tadpoles. This is demostrated for both the control experiments and the exposure experiments (10µg/L atrazine for 24 hour period). N= number of tadpoles, T= Paired T-test, df= degrees of freedom, P <0.05, P*=Wilcoxon Signed Ranks Test 0.05, W= Wilcoxon statistic. The Paired T-test and Wilcoxon signed rank test statistics were calculated by the difference in time spent (s) on kin side during the controls for all the individuals per family to the time spent (s) on kin side during the exposure experiments for all the individual per family.

		control					exposure							
		Mean time spent (s)		Standard error			Mean time spent (s)		Standard error					
		Kin side	Nonkin side	Kin	Nonkin	N	Kin side	Nonkin side	Kin	Nonkin	N	T	df	P
Outbred Family	F1	340.5	319.5	17.5	17.5	30	346.8	313.2	16.6	16.6	30	-0.29	29	0.77
	F2	361.6	298.4	29.5	29.5	30	275.8	384.2	33.9	33.9	30	2.18	29	0.04
	F3	329.6	330.4	23.8	23.8	30					0		0	
	F4	308.0	352.0	29.4	29.4	30	313.5	346.5	34.6	34.6	30	-0.11	29	0.92
	F5	349.1	310.9	13.1	13.1	30	285.6	374.4	20.0	20.0	30	2.36	29	0.03
Inbred Family	FF	330.5	329.5	20.4	20.4	30	328.0	332.0	7.2	7.2	30	0.11	29	0.91
	JJ	349.3	310.7	21.8	21.8	30	328.0	332.0	9.6	9.6	30	0.87	29	0.39
	GG	301.8	358.2	20.5	20.5	30	324.7	335.3	11.8	11.8	30	-1.07	29	0.29
	FFb	331.0	329.0	29.4	29.4	30	306.3	353.7	14.8	14.8	30	0.70	29	0.49
	JJb	305.0	355.0	13.4	13.4	30	324.3	335.7	19.4	19.4	30	-0.77	29	0.45
											N	W	P*	
Inbred - isolated Family	FF	343.7	316.3	21.1	21.1	30	334.3	325.7	11.2	11.2	30	252	0.70	
	JJ	298.8	361.2	16.4	16.4	30	347.5	312.5	21.3	21.3	30	164	0.16	
	GG	330.6	329.4	16.5	16.5	27	355.7	304.3	19.2	19.2	27	142	0.26	
	FFb	326.3	333.7	11.6	11.6	27	328.0	332.0	17.1	17.1	27	201	0.78	
	JJb	356.1	303.9	23.2	23.2	28	353.4	306.6	21.3	21.3	28	228	0.58	

Paired t-tests and Wilcoxon signed-rank tests (MINITAB 14) were used to determine whether there were any differences in time spent (s) on the kin side of the tank during the control experiments (for all of the individuals per family) and the time spent (s) on the kin side of the tank during the exposure experiments (for all the individual per family) (i.e. kin control – kin exposure).

Table 3 Median data used to calculate the Wilcoxon signed rank test for the isolated inbred tadpole families for control experiments and exposure to 10 µg/L atrazine for 24 hour, for both kin and nonkin side preference.

		Control		Exposure	
		Kin side	Nonkin side	Kin side	Nonkin side
Inbred - isolated Family	**FF**	336.6	323.4	341.5	318.5
	JJ	323.3	336.7	334.7	325.3
	GG	317.2	342.8	323	337
	FFb	328.8	331.2	320.8	339.2
	JJb	339.3	320.7	336.3	323.7

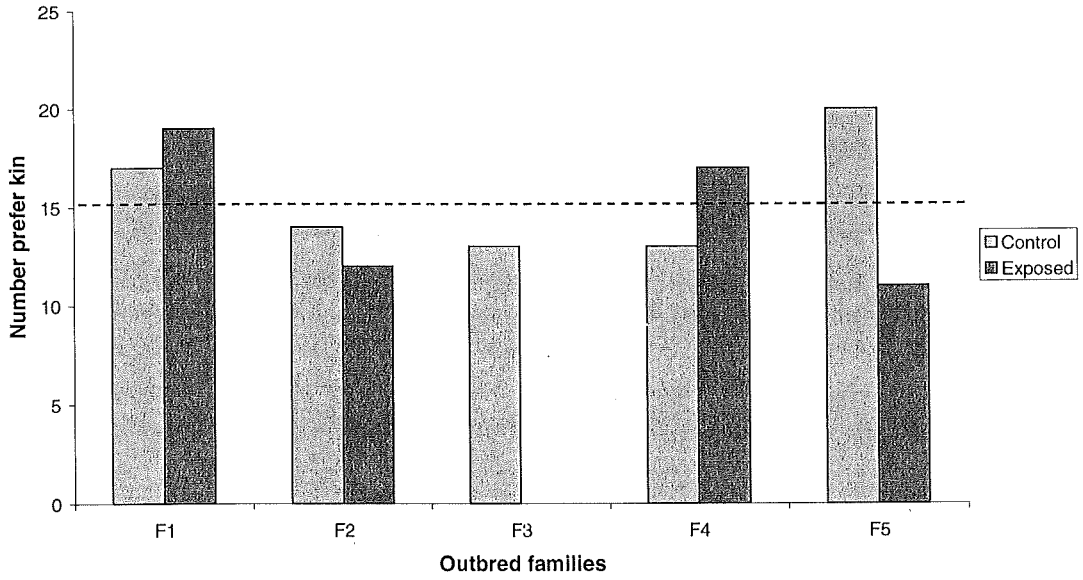

Figure 14 Number of outbred tadpoles that prefer kin before and after exposure to 10 µg/L of atrazine. N= 30 for all families and for control and exposure (dotted line is probability of random)

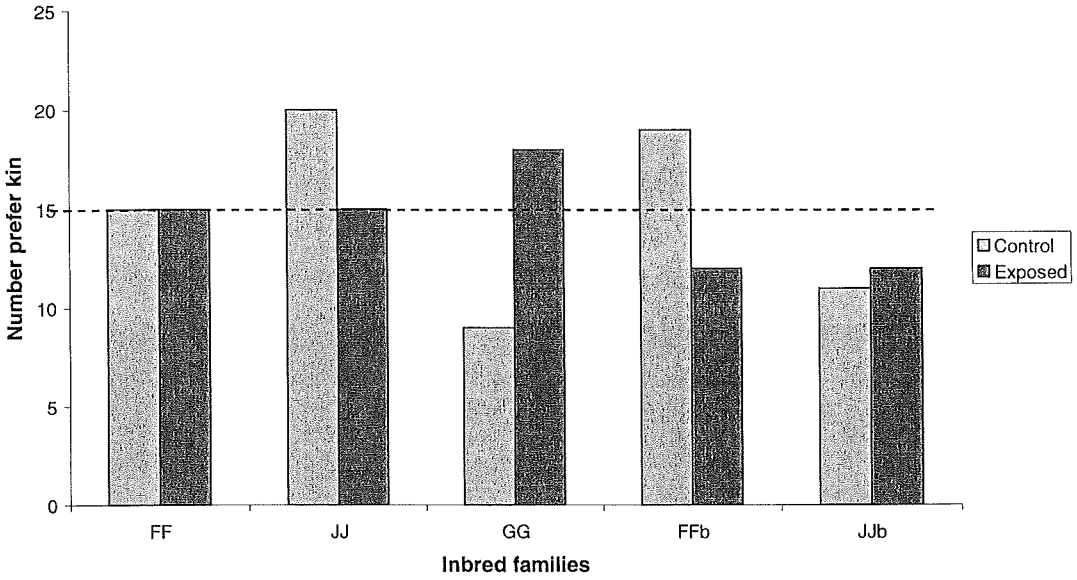

Figure 15 Number of inbred tadpoles that prefer kin before and after exposure to 10 µg/L of atrazine. N= 30 for all families and for control and exposure. (dotted line is probability of random)

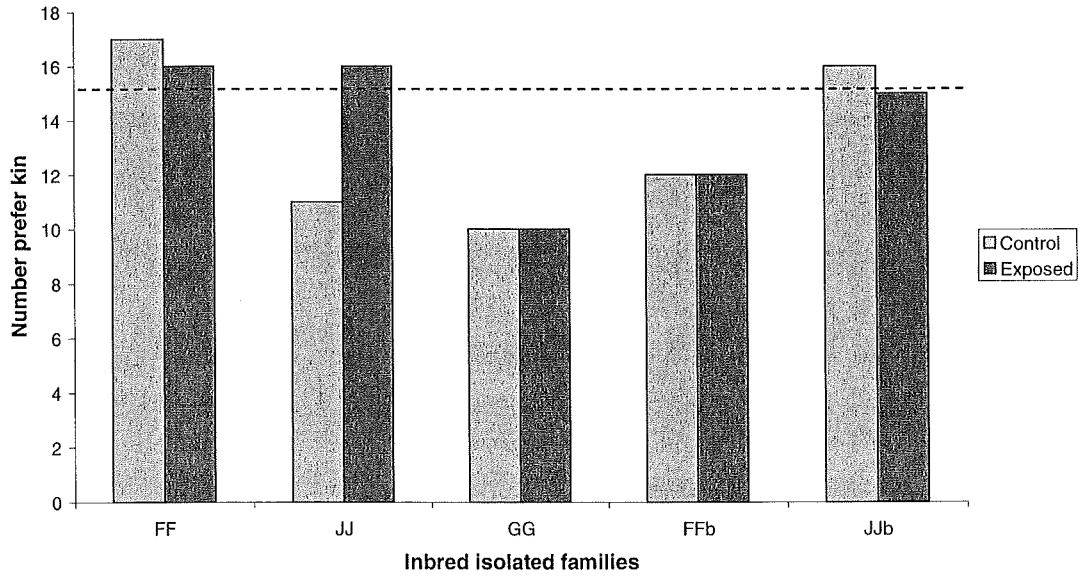

Figure 16 Number of isolated inbred tadpoles that prefer kin before and after exposure to 10 µg/L of atrazine. N= 30 for families FF and JJ for control and exposure, N= 27 for families GG and FFb for control and exposure and N= 28 for family JJb for control and exposure. (dotted line is probability of random)

3.4 Discussion

Amphibian populations have been declining worldwide (Houlahan *et al.*, 2000; Kiesecker *et al.*, 2001) and various explanations for this decline have been proposed, including the effects of parasites (Daszak *et al.*, 1999), disease (Daszak *et al.*, 1999), global warming (Pounds *et al.*, 1999) and toxicants (Davidson et al., 2002). I considered a particular potential impact of toxicants on *Xenopus laevis.* Instead of considering lethal effects, I was interested in whether toxicants have sub-lethal effects that might be contributing to the decline of amphibians.

With extensive use of toxicants worldwide (Aspelin, 1997), there are reasons to suppose that amphibians are not only being directly affected, but also indirectly by spray runoffs into waterways and downwind application (Davidson et al., 2002). Sublethal dosages of toxicants are known to affect endocrine function (Park et al., 2001; Hayes et al., 2003), fertility (Tavera-Mendoza et al., 2002a; Tavera-Mendoza et al., 2002b) and development (Larson et al., 1998).

Atrazine, a herbicide, has been shown to affect the development of gonads and the physical development of the maturing animal (Hayes et al., 2003; Hayes et al., 2002a; Larson et al., 1998; Tavera-Mendoza et al., 2002a; Tavera-Mendoza et al., 2002b). The concentration at which toxicants are present in the environment from indirect application, although lower than the direct application strength, might nonetheless be inflicting significant, if not more damage to the amphibian population.

My results showed an interesting trend. The tadpoles exposed to atrazine were the same individuals that were tested for kin recognition in the previous chapter and they were numbered, making direct comparisons possible between the controls and the exposures.

The tadpoles' exposure to 10 μg/L of atrazine for 24 hours was established by conducting pilot studies using 20 μg/L atrazine for 24 and 48 hrs and 10 μg/L atrazine for 12, 24, 48 hrs and finding that 20 μg/L atrazine for 24 and 48 hrs seemed to have an adverse effect on the movement of the tadpoles (i.e. they became sluggish). I decided, therefore, not to use this concentration because I wanted to minimise physical side effects. Trialling the 10 μg/L atrazine for 12 hrs really did not give much of a response and 48 hrs exposure again seemed to make them more sluggish than I wanted. The assay for which I opted for was 10 μg/L of atrazine for 24 hrs. This was an ecologically relevant dose and a realistic time period. (All further mention of atrazine exposure is at 10 μg/L of atrazine for 24 hours.)

The outbred and inbred families after exposure to atrazine preferred to spend their time on the nonkin side of the tank during the tests, compared with the trend seen in the controls, of kin preference. The isolated inbred tadpoles were the exception to the trend; they showed an increase in kin preference after exposure to atrazine, compared with the control tests.

With reference to the Chapter 2 and reanalysing the tadpole results with respect to an honours project studied by M. Locker (Harvard University,1989), the exposure data were reanalysed the same (investigating 2 and 3 divisions for both the initial 10 min and the full 60 min) and I came to the same conclusion as the control experiments, that the data showed the strongest trends when conducted for the full 60 minutes and with only two divisions.

These results suggest a trend for kin preference during the controls and that when the tadpoles are exposed to atrazine there is an 'active' avoidance of kin.

This finding is the opposite of what I originally predicted would happen. My prediction was that, when the tadpoles were exposed to a harmful herbicide, the tadpoles' chemical

communication system would break down, interfering with the tadpoles' ability to choose where to go (i.e. they would not be able to read the chemical cues coming from kin). However, my findings suggest that they were actively avoiding kin, (i.e. spending more time on nonkin side). It is tempting to suggest this finding was evidence that tadpoles, when sick, altruistically avoid their kin to avoid making them sick. Then the behavioural response to the toxicant might prevent a whole brood becoming contaminated and we might predict that the healthy tadpoles also avoid contaminated individuals. However, no firm conclusions about this hypothesis are possible at this stage.

The isolated tadpoles, however, did not show kin avoidance, but instead a stronger kin preference when the control and the other two groups were compared. An increase in stress might explain this change (from isolation and food deprivation). Possibly, the level of contamination was not as threatening as the level of stress the tadpole had experienced, and therefore, when stressed, the advantage gained by associating with kin outweighs for this individual the disadvantage to its inclusive fitness of contaminating or spreading sickness to its kin.

In the wild, amphibians that are contaminated by atrazine (and possibly sublethal concentrations of other toxicants) might experience a decrease in the individuals inclusive fitness and/or there could be an increase in the death rate. However, if there is active avoidance between the contaminated individual and the rest of the brood, the inclusive fitness of the brood should remain high. But, the individual that is isolated from the kin group could face a decrease in its ability to search for food and avoid predators because of the benefits it will be losing of kin aggregation (Waldman & Adler, 1979).

Initially, the assay used in the tests I conducted needs to be modified so that the tadpoles are restricted to using chemical cue alone without the possible aid of visual cue. This could be achieved by placing a plastic sheet with small holes in it between the nets and the subject arena.

Further studies need to be conducted to gain a better insight into the sublethal effects of a toxicant such as atrazine. One study could involve exposing the tadpoles held in the stimulus nets and having the subject 'healthy', with the aim to see whether the subject shows signs of kin avoidance of contaminated tadpoles. Another test could be identifying whether the chemical cue is disrupted by the toxicant in the water, having both the subject and the tadpoles in the nets 'healthy' and adding the atrazine to the water.

I believe it would be important to exposure the adult frogs to atrazine (and other toxicants) to investigate whether the toxicants disrupt their chemical communication system. The experiments in Chapter 2 could be adapted so the subject is exposed to atrazine and given 'healthy' female treatment water. Another test would be to have a 'healthy' subject but having the treatment female previously exposed to atrazine and then placed in the treatment water (before testing the subject); and lastly have the subject and the treatment female 'healthy' and add the atrazine to the test water. These three testing procedures would give an understanding of whether the mechanism affected by atrazine is perception, production or transmission, accordingly.

The tadpole results suggest there is a behavioural response even at low levels of toxicant exposure. The kin preference response is strengthened but also reversed, because they altruistically avoid kin, with atrazine exposure. The results also suggest that there is a balance

between the threat that stress could give versus contamination, it was evident that stress was more of a threat than 24 hour of atrazine exposure (10 μg/L).

3.5 Reference list

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Chapter Four

Organic Interceptor vs. Roundup Renew

4.1 Introduction

Due to an increase in awareness of amphibian decline worldwide, many studies were conducted to gain an understanding of the indirect effects that toxicants (herbicides, pesticides and insecticides) have on affected animals (Houlahan et al., 2000; Kiesecker et al., 2001).

Studies have shown toxicants can disrupt the behaviours of exposed animals. Exposured freshwater organisms and amphibians have shown decrease in the feeding rates (Hussein.S.Y. *et al.*, 1996), mobility (Hussein.S.Y. et al., 1996; Gopal et al., 1981) and mating success (Park et al., 2001; Tavera-Mendoza.L. et al., 2002b; Tavera-Mendoza.L. et al., 2002a; Hayes et al., 2002a). Toxicants can interfere with their maturation period (Larson et al., 1998; Brown Sullivan & Spence, 2003; Hecnar, 1995) and normal development (Tavera-Mendoza et al., 2002a; Hayes et al., 2002a; Hayes et al., 2003; Hayes et al., 2002b; Harris et al., 2000; Tavera-Mendoza et al., 2002b). These toxicants can cause these behavioural and physical alterations at sub-lethal concentrations.

Toxicity studies are conducted on test species that are representative of receptor species that needs to be protected. Rats and rabbits are used as surrogates to evaluate the toxicity of chemicals to humans.

A bioindicator is an organism that is used to monitor and characterise the health of a specific environment (Phillips, 1990). Bioindicators are also viewed as either structural entities such as sentinel species or they can be considered functionally as biological effects endpoints at higher levels of organisation (Adams & Tremblay, 2003). Amphibians have been used as bioindicators as they are likely to be exposed to environmental contaminants due to their biphasic life cycles, early aquatic followed by terrestrial stages (Wilbur et al., 1990; Stebbins & Cohen, 1995). Amphibians have a semi-permeable skin making them susceptible to toxicant exposures and disease (Stebbins & Cohen, 1995; Mann et al., 2003).

Due to an increase in awareness of the effects of toxicant exposure on animals, there has been an increase in the number of studies conducted to investigate the chemistry of the toxicants and how they can be improved to be less harmful to animals. Clearly, control agents are required to control and possibly eradicate pests and weeds, but these ideally should be chosen so that they are safe for both humans and non-target organisms. One mode of thinking is to use organic instead of synthesised products.

Organic labels are meant to promote the image of non-toxic product containing safe natural ingredients devoid of negative side effects. However, this can be a false indication of safety as some natural compounds may be more dangerous than synthesized ones, eg sodium monofluorocitrate, 1080.

Organic Interceptor (Certified Organics, New Zealand) is an organic herbicide containing pine extract as active ingredients. Organic Interceptor has been certified

organic by BIO-GRO New Zealand, AgriQuality Certenz, Biological Farmers of Australia (BFA) and National Association for Sustainable Agriculture, Australia Ltd (NASAA) (Certified Organics, 2002). Organic Interceptor kills plant material by disrupting normal membrane permeability causing dehydration. It is effective by direct contact on plants within one hour of treatment, quickly deactivated on the soil, and biodegraded within 48 hours. Organic Interceptor is non-selective and controls anything from thistles to broadleaf. The recommended application rate is 20 % in water.

Roundup Renew herbicide is a synthesized product and has an active ingredient of glyphosate, an isopropylamine (IPA) salt. A variety of glyphosate-based formulations are available in over 100 countries (Williams et al., 2000). Roundup Renew is used for the control of annual, perennial and aquatic weeds. Roundup kills the plants by using the glyphosate to inhibit plant growth by inhibiting enzyme enolpyruvylshikimate phosphate synthesis which results in inhibiting the production of aromatic amino acids (Williams et al., 2000). Roundup is primarily broken down in the environment by microorganisms (Williams et al., 2000). The recommended dose of this herbicide is 1% solution and with advice to follow the application instructions. There are many different formulations of Roundup all over the world.
I conducted an experimental comparison between two herbicides, one organic and the other synthesized, namely Organic Interceptor and Roundup Renew, respectively.

There is no published information in the scientific literature on the toxicity of Organic Interceptor. Waiheke Island Community Awareness of Pesticides (CAP), and

Waiheke ME, Multiple Chemical Sensitive, and Chronic Fatigue Syndrome Group on behalf of Certified Organics Ltd, conducted a trial investigating indirect effects of Organic Interceptor on humans, and found no immediate or postponed illness or any bad effects from the spraying, also, they observed no adverse effect on any insect life (Certified Organics Ltd website – Testimonials - http://www.certified-organics.com/index.cfm/pid_12).

Several studies have been conducted to investigate the effects of Roundup and the active ingredient glyphosate on different animals. Howe *et al* (2004) examined the toxicity of glyphosate technical, glyphosate and polyethoxylated tallowamine (POEA) surfactant that is in Roundup Original and five other glyphosate based herbicides on four different frog species. They discovered that the formulated glyphosate pesticide that did not have the POEA surfactant present and glyphosate alone was less toxic than pesticides that did include POEA surfactants.

A surfactant is a product that is added to (in this case) a herbicide to help the active ingredient to dissolve in water and/or to disperse easily and stick to and be absorbed into the plants leaves. Often it is the case that the surfactant is the more harmful product in the formulation (Perkins et al., 2000).

Studies have been conducted investigating the affect of Roundup on fish. Hildebrand *et al.*(1982) discovered that Roundup exposure at different concentrations in the field and laboratory have a very similar 96 hour LC50, meaning there was no environmental interaction effect, such as weather effects or interactions with other

4.2 Method

Two families of *X. laevis* tadpoles were bred using wild caught animals from South Africa. Lutenizing Hormone Releasing Hormone (LHRH) (Argent Laboratory, 8702, 1-52nd Ave, North East, Redromd, WA, 98052 USA) was injected at 100 µg into the female on the day of the breeding to ensure the female was receptive and therefore a successful breeding and the male required no LHRH (Kelley, 1982). LHRD was injected into the dorsal lymph sac (Kelley, 1982). The broods were divided into separate tanks holding 150 tadpoles to avoid overcrowding and to increase the rate of maturation. The clear polypropylene tanks were 360 cm by 465 cm by 170 cm, with 30 litres of water (plastic 5). Later, when the tadpoles had increased in size and were free swimming and feeding, they were split up again into more tanks holding about 60 tadpoles, again to help increase growth rate and maturation. The tadpoles were raised at 22 °C, in a temperature-controlled room. An automated day and night cycle maintained the light hours between 0800 to 2000 and the dark hours between 2000 to 0800. They were fed strained ground nettle ad lib. This continued until they were 27 days old and ready to test. The tadpoles were staged to the *Xenopus laevis* stage 48-50 (Niewkoop & and Faber, 1975).

The experimental assay involved one-litre non-toxic plastic (from TECPAK, Packaging Innovators, Christchurch, New Zealand), classification plastic 5, containers which held one tadpole and 500mls of solution. The experiment was conducted in natural photoperiod with a semi-regulated temperature.

The Organic Interceptor concentrations used in ml/L were 0.0005, 0.001, 0.003, 0.009, 0.015, 0.02, 0.03, 0.04, 0.05. Roundup Renew concentrations used in ml/L were 0.072, 0.36, 0.72, 1.00, 1.25, 1.5, 1.8, 3.6.

The concentrations were determined by starting with a percentage of the recommended dose and altering the dosage level according to the mortality results.

The control experiments were held in aged tap water (Christchurch city water) that had been run through a 5 μ paper filter. The experimental concentrations were made using the same sourced aged tap water.

Each herbicide concentration was made the day of the experiment, to avoid any aging or disruption of the herbicides. The water quantity was measured using a two litre plastic measuring cylinder and the concentration of the herbicide was measured using a Gilson Pipetman (P100 and P200), both were emptied into a ten litre bucket and stirred using a magnetic stirring rod (Corning, Hot plate stirrer, the stirring rod was 3 cm) for no more than five minutes. The final concentration of 500 ml was measured using a one-litre Pyrex measuring cylinder and emptied into the experimental container.

There were 20 replicates at each concentration and this was repeated for two families. The containers were arranged into blocks of Organic Interceptor and Roundup Renew. Within the blocks, the concentrations of the herbicides were randomized.

Every 24 hours a count of the mortalities at each concentration was recorded. I determined a tadpole to be dead if it did not move when prodded by the blunt end of a rod. The trial lasted for 4 days, a period of 96 hours. The room temperature was 20°C

±1°C and the pH for the different concentrations of both Organic Interceptor and Roundup Renew was 7.8 ± 0.1. No animals were fed during the experimental period.

4.3 Results

Xenopus laevis tadpoles showed a high sensitivity to the exposure of Organic Interceptor and Roundup Renew after a 96 hour period. The concentration of Organic Interceptor that kills 50 percent of these tadpoles is at a lower concentration than the Roundup Renews' LC50.

Organic Interceptor has a recommended dose of 20 % solution. My results suggest a LC100 of 0.05 ml/L concentration and a LC50 of 0.0255 ml/L concentration (data were log transformed data were analysed using the PROBITANALYSIS procedure in the statistical package GenStat.) (see Table 4).

Table 4 Table describing the LC100 and LC50 of Organic Interceptor and Roundup Renew after 96 hours of tadpole exposure, this is compared to the recommended dosage.

Herbicide	Recommended dose (ml/L)	LC100 (ml/L)	Difference between recommended dose and LC100	LC50 (ml/L)	Difference between recommended dose and LC50
Organic Interceptor	200	0.05	4000 times lower	0.02553	7833.9 times lower
Roundup Renew	10	1.8	5.6 times lower	1.1254	8.9 times lower

With reference to Figure 17 (the data is log transformed) and Table 5, the tadpoles exposed to Organic Interceptor below 0.01 ml/L concentration had variability in number of deaths but with all mortalities below 10 %, with the exception of one outlier. From 0.01 to 0.02 ml/L the mortalities roughly doubled and are up to 27 %

dead. At a concentration of 0.03 ml/L the mortality rate is almost double of the last point, with the percentages of deaths to 40 %. There is a big jump between 0.03 ml/L and 0.04 ml/L where the percent of mortalities rises from 40 % to just over 80 % of all the tadpoles dying at this concentration. Lastly, at 0.05 ml/L we have LC100, a 100 % death rate. LC50 (data were log transformed and were analysed using the PROBITANALYSIS procedure in the statistical package GenStat.) was estimated at 0.0255 ml/L.

Generally, the majority of the deaths occurred within the first 24 - 48 hours and the tadpoles that died during the experiment were left until the experimental run was complete. At the end of the experimental period the tadpoles that had died were found dehydrated and stuck to the container and had started to disintegrate. The tadpoles that survived seemed sluggish with low motility.

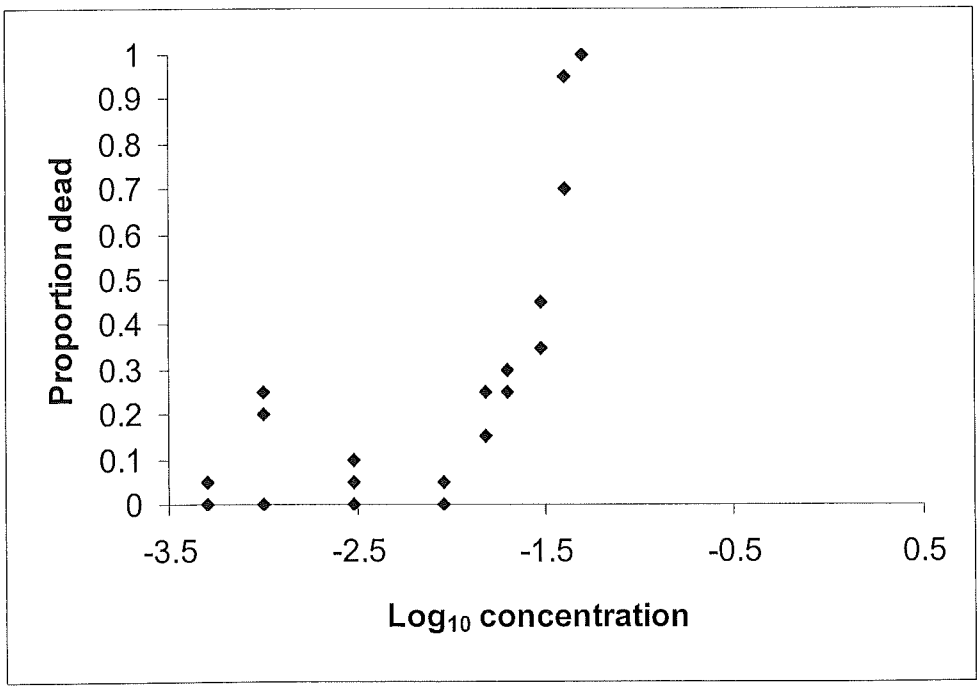

Figure 17 Log transformation of the number of tadpoles that died with exposure to different concentrations of Organic Interceptor after 96 hours.

The LC100 for Roundup Renew is 1.8 ml and the LC50 is 1.125 ml/L (data were log transformed and analysed using the PROBITANALYSIS procedure in the statistical package GenStat.) (see Table 2). Roundup Renews' mortality rate seems to have a slightly different trend than Organic Interceptor (see Figure 18). When the concentration of Roundup Renew is between 0 and 1 ml/L the percentage of deaths fluctuates from under 5 % to just over 20 %, with the exception of one outlier. With a concentration from 1.25 ml/L to 1.8 ml/L there is a steep and constant increase in the percentage of deaths, all the way to 100 % mortality at 1.8 ml/L.

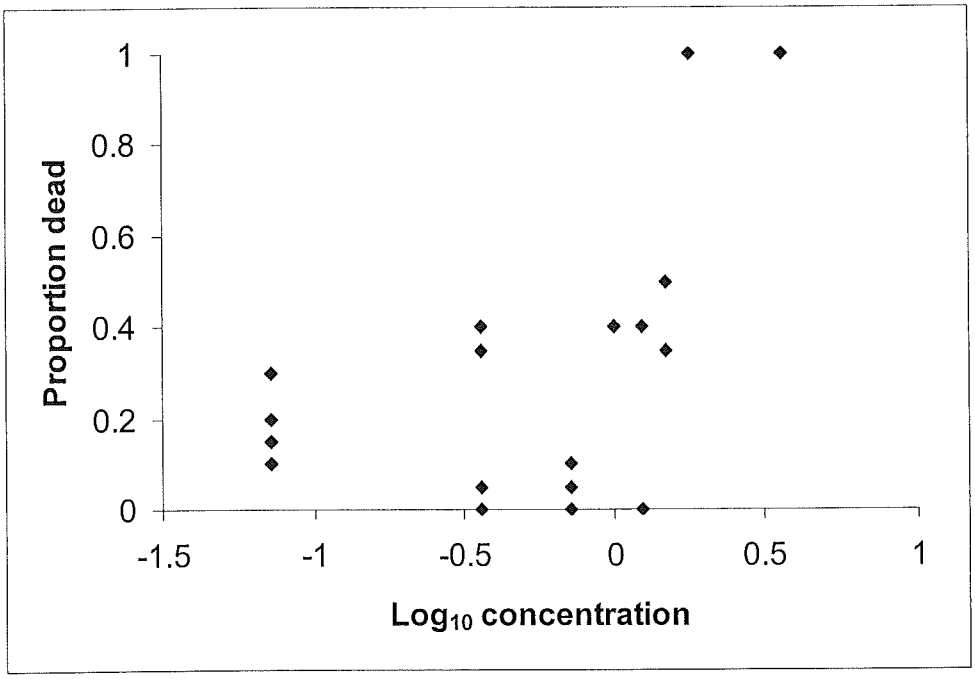

Figure 18 Log transformation of the number of tadpoles that died with exposure to different concentrations of Roundup Renew after 96 hours.

The majority of Roundup Renew deaths were also within the initial 24 – 48 hours of their exposure. The tadpoles that survived the experiment were more mobile compared to tadpoles in the Organic Interceptor treatment, and there were also no signs of disintegration seen in the remaining dead tadpoles.

Fewer than 5% of the control tadpoles died and in some trials there were no deaths. The two families that were used in the experiment showed very little variation in mortality results for both the Organic Interceptor and the Roundup Renew experiments (see Table 5& 6).

Table 5 The number of deaths each family incurred at each trial and at each concentration of Organic Interceptor after 96 hours of exposure.

Organic Interceptor				
	29/08/2003			
Concentrations ml/L	Number (Dead)			
	Family 1 (total 20)	Family 2 (total 20)	TOTAL	%
0.001	4	5	9	22.5
0.003	0	0	0	0
0.0005	0	1	1	2.5
	12/11/2003			
Concentrations ml/L	Number (Dead)			
	Family 1 (total 20)	Family 2 (total 20)	TOTAL	%
0.001	0	0	0	0
0.003	2	1	3	7.5
0.009	1	0	1	2.5
0.05	20	20	40	100
	6/04/2004			
Concentrations ml/L	Number (Dead)			
	Family 1 (total 20)	Family 2 (total 20)	TOTAL	%
0.015	3	5	8	20
0.02	6	5	11	27.5
0.03	9	7	16	40
0.04	19	14	33	82.5

studying toxicants to find out how lethal they are to different groups of bioindicator animals. Commercial products have been altered and some even taken off the market to make them less harmful to potentially exposed animals, for example DDT has been removed (Kehoe & Jacobson, 2003). New products have been released claiming to be 'safer' for the environment, and some of these are organic products.

The general consensus is that an organic product is safer for the environment and animals than a synthetic product (personal communication T. Bruce). The results from this study seem to strongly disagree. The recommended dose of Organic Interceptor, an organic product, is 20 % solution and is deemed safe for the environment. Roundup Renew, a synthetic product, has a recommended dose of 1 % solution. When exposing *X. laevis* tadpoles to concentrations of Organic Interceptor that was hundreds of times lower than the recommended dose for a period of 96 hours, there were still huge mortalities. According to the LC50 value, when only 50 % of the tadpoles die when exposed, the minimal concentration for Organic Interceptor is 0.0255 ml/L. This LC50 value is thousands of times lower than the recommended dose (see Table 4). In comparison, Roundup Renew has a LC50 value just over eight times lower than the recommended dose (see Table 4). These results clearly indicate that Roundup Renew, a synthetic product, is less harmful to tadpoles, a bioindicator, than Organic Interceptor. This finding is new and exciting I have struggled to find publications on organic herbicides and any toxicity testing on amphibians or bioindicators. I believe that there should be thorough research on the effects the product could have on possible animals that might be exposed, either directly or indirectly, on a product before it goes into the consumers market.

There has been no thorough research (that I could find) conducted to investigate the toxicity of Organic Interceptor on amphibians or bioindicators.

Whereas, Roundup has been researched and shown to be a reasonably safe product at application strength. Mitchell *et al.* (1987) studied coho salmon smolt (*Oncorhynchus kisutch*) and found there was no adverse effect of Roundup on the smolt. Also, studies on rainbow trout discovered that 'operational application' and 100 times higher concentrations than the field application of Roundup should not be detrimental to these fish (Hildebrand et al., 1982). In conclusion to a extensive study by Williams *et al.* (2000) it was found that Roundup and its active ingredient, glyphosate, should be of no risk to humans. Hildebrand *et al.* (1980) and Sullivan *et al.* (1981) both discovered Roundup had little effect on *Daphnia* and diatoms respectively, but that Roundup may have a longer lasting effect on the population or other trophic levels such as the surrounding community, predators and prey of the amphibian.

Organic Interceptor and Roundup Renew both use different mechanisms to achieve their objectives of killing plant life. Organic Interceptor uses a non-targeted method. It dehydrates whatever is in its pathway, by breaking down the cell walls. Roundup Renew on the other hand uses a targeted mechanism, where it breaks down enzymatic processes to stop the growth of the plant. This mechanism is exclusive to plants because aromatic amino acids biosynthesis does not occur in this way in the animal kingdom (Williams *et al.,* 2000). The different mechanisms these two herbicides have is a possible explanation for the difference in toxicity and also a reason why the dead tadpoles decomposed differently, with Organic Interceptor exposed tadpoles

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Chapter 5

5.1 Discussion

There have been numerous hypothesis suggested for the global decline in amphibian populations (Kiesecker et al., 2001; Houlahan et al., 2000), including disease (Daszak et al., 1999), global warming (Pounds et al., 1999) and toxicants (Davidson et al., 2002). My objective was to examine especially the impact of toxicants on amphibians. Potential sublethal effects were of particular interest, and I focused on repercussions on amphibian of exposure to a common chemical communication process. The study animal I chose was a common species of frog, *Xenopus laevis,* and I considered how atrazine might disrupt this species chemical communication process.

With the worldwide usage of toxicants per annum being nearly 10 billion kg (USA billion) (Aspelin, 1997), there is a clear need for an understanding of how these toxicants affect the environment. In this context, the decline in amphibian populations is particularly alarming. Amphibians have a biphasic lifestyle and other traits that may make them especially suitable as bioindicators of environmental health (Phillips, 1990; Wilbur et al., 1990; Stebbins & Cohen, 1995).

It is not just the application dose that is worrying. We also need to understand the sublethal negative effects of prevalent lower concentrations. Even when the application strength has been diluted by rainfall, dilution in streams, ponds and waterways, what remains may be just as worrying and negative (Davidson et al., 2002). Sublethal effects on amphibians include hermaphroditism (Hayes et al., 2002a; Hayes et al., 2003), developmental problems (Larson et al., 1998), fertility problems (Tavera-Mendoza et al., 2002a; Tavera-Mendoza et al., 2002b) and disruption of the endocrine system (Park et al., 2001; Park & Propper, 2002;

Hayes et al., 2003). Not only might exposure to the toxicant have direct physiological effects on the animal but it might also increase the amphibian's vulnerability to stress. This may in turn increase mortality by a factor of at least two (Relyea & Mills, 2001). In the long term, side effects of toxicants on a population can be lethal because of effects on the whole community and across trophic levels. For example, predators may be poisoned by feeding on contaminated prey (Hildebrand et al., 1980; Stebbins & Cohen, 1995).

Finding a herbicide that kills a target plant without causing harm to the animals in the environment is a serious challenge, but organic products, as an alternative to synthetic products, are often advocated on the basis of the assumed safety of these alternative. However, that should not be simply assumed.

In my own research the LC50 of an organic herbicide versus a synthetic herbicide (Organic Interceptor and Roundup Renew, accordingly), I discovered that a synthetic herbicide, Roundup Renew, was the less harmful of the two. Organic Interceptor, at a dose literally thousands of times lower than the recommended dose of 20 %, killed 100 % of the *Xenopus laevis* tadpoles exposed to it, generally within a 24- 48 hour period. This product kills the plants by dehydration of the cell walls (Certified Organics, 2002) and dehydrations of the cells walls is a likely explanation for its effect on *Xenopus laevis*. However, the mechanism by which Roundup Renew works is exclusive to plants (Williams et al., 2000). The results from the experiments showed that 100 % of the tadpoles were dying at only about 5 times lower than the 1 % solution recommended dose.

At least in this instance, the synthetic herbicide, appears to be safer than the organic product. Yet I have struggled to find any publications of toxicity

testing on amphibians or other bioindicators using organic herbicides. My impression is that many people must simply assume that organic products are safe simply because they are organic. This may be a dangerous misconception and such herbicides should not actually be put on to the market before their toxicities have been thoroughly considered thorough experimentation. With a synthetic product like Roundup we can rely on there having been multiple studies conducted on its toxicity on the basis that these studies are of which we can judge how safe it will be for animals that might be exposed, including, fish (Mitchell et al., 1987; Hildebrand et al., 1982), tadpoles (Smith, 2001), frogs (Howe et al., 2004), diatoms (Sullivan et al., 1981) and *Daphnia* (Hildebrand et al., 1980). Generally, objective information about the safety of organic herbicides is simply not available.

Sublethal effects of toxicant may tend to be overlooked, and the stages required for evidence may often be more involved than for lethal effects. For my research I initially investigated whether *Xenopus laevis* adults and tadpoles actually do communicate chemically and then I investigated the impact atrazine had on the chemical communication system of *Xenopus laevis* tadpoles (due to unavailability of adult frogs).

Initially, for the adult *Xenopus laevis* frogs, I was looking evidence of chemical cues being involved in courtship behaviour, but what I found instead was evidence of chemical cues influencing female – female interaction. It may be that females are especially territorial and that chemical cues function in the context of territoriality. In the work on adult frogs, the male subjects showed little response when exposed to female treatment or to male treatment water. Perhaps males are not so territorial as females and makes them not prone to responding to these chemical cues. Males may need a secondary cue, such as the physical presence of another frog, before they will respond even when the chemical cue is present. Males may

normally migrate away from the breeding area, or they may be generally more nomadic than females, whereas the female may not migrate as far and she may be more territorial. Females may have evolved a strategy of responding more readily than males to chemical cues alone. This hypothesis may be related to Fishers hypothesis "Dear enemy" (1954).

Something similar may apply to the red-backed salamander (*Plethodon cinereus*). Females of this species are more responsive than males to cues from other frogs and this appears to be related to territory being especially important for reproductive success and survival of females (Horne & Jaegar, 1988).

My results provided no evidence that males of *Xenopus laevis* communicate chemically. However, I tentatively conclude that females of *Xenopus laevis* communicate chemically. Unfortunately, I did not have time to proceed to the next step and expose these adults females to atrazine. Whether atrazine has any adverse effects on the chemical communication system of the adults of *Xenopus laevis* remains uncertain.

However, I did go further in this work using tadpoles. It has been argued that kin recognition is important to tadpoles because it increases the inclusive fitness because schooling increases the food availability and also using alarm signals for warning of others of the presence of a predator through alarm signals is more beneficial to inclusive fitness when in a kin group (Hews & Blaustein, 1985). In my results there was a trend for kin preference when outbred, inbred and isolated inbred tadpoles of *Xenopus laevis* were tested.

Atrazine, a herbicide that kills the target plant by inhibiting photosynthesis (Solomon et al., 1996), is classified as a class C compound and has a maximum contamination level of

3 $\mu g/L^{-1}$ in America (Shipitalo & Owens, 2003; Wolf & Nowak, 1996). There have been many studies on the toxicity of atrazine and the side effects of atrazine (Laws et al., 2000; Hussein et al., 1996; Harris et al., 2000; Hayes et al., 2002a; Hayes et al., 2002b; Brown Sullivan & Spence, 2003; Solomon et al., 1996). Still other studies have considered the effects of atrazine on *Xenopus laevis* tadpoles. In these studies, little effect on growth of the tadpoles or no effect on mortality were found. However, gonadal abnormalities and plasma testosterone levels were affected at all doses tested (Hayes et al., 2002a; Hayes et al., 2002b). The implication that atrazine may interfere with the endocrine system (Hayes et al., 2002a) intrigued me. Endosulfan disrupts endocrine function and I was interested whether atrazine might affect the chemical communication systems of amphibians similarly (Park et al., 2001).

I used the kin preference data as a baseline and exposed the same tadpoles to atrazine. Then I observed whether there were any behavioural changes. I had previously hypothesised that atrazine would inhibit the endocrine system. On the basis of previous studies on herbicides (Park et al., 2001), I predicted that the presence of kin in a net would not alter the subject tadpoles response. However, my results suggested that the tadpoles did respond to kin after exposure to atrazine but unexpectedly they seemed to be actively avoiding the kin. The isolated inbred tadpoles were an exception. A hypothesis suggested by these results is that the tadpoles detected their own contamination and then altered their behaviour so as to avoid spreading the toxins to their kin. Isolated tadpole did not respond in this manner.

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